

**Analysis of the Sec18 protein from
Saccharomyces cerevisiae.**

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*I dedicate this thesis to my parents
for their love and support over the years.*

Declaration

This study was carried out under the supervision of Dr. Alan Boyd in the Department of Biochemistry, University of Edinburgh between October 1990 and February 1994.

The experimental work carried out in this thesis, unless otherwise stated, is my own; and this manuscript has been composed by myself.

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Abstract

The Sec18 protein (Sec18p) of the yeast *Saccharomyces cerevisiae* has been identified as a component involved in the vesicular transport of proteins through the secretory and endocytotic pathways. Sec18p is a homologue of the mammalian protein NSF which has been shown, using a number of *in vitro* transport assay systems and affinity purification procedures, to interact with other proteins in a multisubunit protein complex. This complex is thought to mediate the fusion of a specific transport vesicle with its respective target membrane allowing the specific targeting of a cargo protein to its final destination. A genetic interaction has been demonstrated between *SEC18* and *SEC17* (encoding the homologue of mammalian α - SNAP) and a number of SNAP receptors have recently been isolated from bovine brain cytosol but no direct interaction has been demonstrated between Sec18p/Sec17p and these proteins within a yeast system.

This work represents two approaches taken with the aim of identifying proteins that interact with Sec18p in the yeast *Saccharomyces cerevisiae*. Isolation of protein complexes was first attempted by affinity purification of a tagged version of Sec18p. The protein was C-terminally tagged with a protein A moiety from *Staphylococcus aureus* containing IgG binding domains. It was hoped that the affinity of protein A for IgG Sepharose could be used to isolate protein complexes that formed *in vivo* with the Sec18p. Although the fusion construct was shown to be active *in vivo*, specific complexing proteins could not be isolated due to the large amount of non-specific binding of yeast proteins to the protein A moiety.

A second genetical approach was used where the *SEC18* gene was randomly mutagenised and yeast cells harbouring these mutagenized genes were screened for a dominant negative phenotype. Dominant negative mutant forms of the Sec18p interfere with the normal function of the wild-type protein *in vivo*. Five such mutants were isolated and classified into two main groups using a number of biochemical and morphological criteria. Class I mutants show a classical secretory mutant phenotype whereas the Class II mutant has a novel phenotype. A number of mutants in which the dominant negative phenotype was suppressed were isolated but the genes responsible for this phenotype could not be identified. It is hoped that alternative strategies can be employed in the future to identify extragenic suppressors of these mutants.

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Finally, I would like to recognise the excellent support of the Wellcome Trust throughout my Ph.D.

Abbreviations

A ₄₉₀	absorbance at 490 nm
bp	base pairs
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
<i>C. albicans</i>	<i>Candida albicans</i>
CGN	<i>cis</i> Golgi network
CHO	chinese hamster ovary cells
COP	coat protein
CPY	carboxypeptidase Y
Da	dalton
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNM	dominant negative mutant
DNP	dinitro phenol
DPAP	dipeptyl aminopeptidase
ECL	enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	diaminoethanetetra-acetic acid
EGTA	1,2 di (2-aminoethoxy) ethane- N,N,N',N'- tetra acetic acid
EM	electron microscopy
EMS	methanesulphonic acid ethyl ester
ER	endoplasmic reticulum
g	relative centrifugal force
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
GlcNAc	<i>N</i> -acetylglucosamine

GTP γ S	guanosine-5'-O-(3-thiotriphosphate)
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	horse radish peroxidase
Ig	immunoglobulin
IPTG	isopropylthiogalactosidase
LCRI	low cytosol requiring intermediate
Man	mannose
min	minute
NEM	<i>N</i> -ethylmaleimide
nm	nanometers
NSF	NEM sensitive factor
OD ₆₀₀	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethanesulphonyl fluoride
RNA	ribonucleic acid
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SDS	sodium dodecyl sulphate
SRP	signal recognition particle
ts	temperature sensitive
TBS	Tris buffered saline
TGN	<i>trans</i> Golgi network

Tris	2-amino-2-(hydroxymethyl) propane-1,3-dio(tris)
UDP	uridine 5'-diphosphate
VSV	vesicular stomatitis virus

Amino acid abbreviations

<i>amino acid</i>	<i>abbreviations</i>	
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamate	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Iso
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

<i>Contents</i>	<i>Page</i>
<i>Declaration</i>	<i>I</i>
<i>Abstract</i>	<i>II</i>
<i>Acknowledgements</i>	<i>III</i>
<i>Abbreviations</i>	<i>IV</i>
<i>Contents</i>	<i>VII</i>
<i>List of figures</i>	<i>XIII</i>
<i>List of tables</i>	<i>XVI</i>

Chapter 1

<i>Introduction</i>	<i>1</i>
<i>1.1 Introduction</i>	<i>2</i>
<i>1.1.1 Targeting of proteins to the endoplasmic reticulum (ER)</i>	<i>2</i>
<i>1.1.2 Translocation of proteins into the ER</i>	<i>3</i>
<i>1.1.3 Processing of proteins within the ER</i>	<i>5</i>
<i>1.1.4 Processing of proteins within the Golgi apparatus</i>	<i>6</i>
<i>1.1.5 Bulk flow of proteins through the secretory pathway</i>	<i>10</i>
<i>1.1.6 Secretory mutants of <i>Saccharomyces cerevisiae</i></i>	<i>11</i>
<i>1.2 Intracellular membrane traffic</i>	<i>13</i>
<i>1.2.1 Cell free transport system</i>	<i>13</i>
<i>1.2.2 NEM sensitive factor (NSF)</i>	<i>16</i>
<i>1.2.3 Soluble NSF attachment proteins (SNAP)</i>	<i>18</i>
<i>1.2.4 Evolutionary conservation</i>	<i>19</i>
<i>1.2.5 20S particle involved in membrane docking/fusion</i>	<i>21</i>
<i>1.2.6 Vesicle SNAP receptors (v-SNAREs) in yeast</i>	<i>24</i>
<i>1.2.7 Target vesicle SNAP receptors (t-SNAREs) in yeast</i>	<i>26</i>
<i>1.3 Regulation of intracellular membrane traffic</i>	<i>27</i>

1.3.1	<i>GTP binding proteins</i>	27
1.3.2	<i>Regulation of vesicle formation events</i>	29
1.3.3	<i>Regulation of intermediate vesicle docking/fusion events</i>	32
1.4	<i>Outline of project</i>	36

Chapter 2

Materials and Methods	37
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Materials

2.1	<i>Chemicals / antibodies / enzymes</i>	38
2.2	<i>Bacterial / yeast strains and plasmids</i>	38
2.3	<i>Bacterial plates / media</i>	38
2.4	<i>Yeast plates / media</i>	39

Methods

2.5	<i>DNA manipulations</i>	39
2.6	<i>Transformation of bacterial and yeast cells</i>	40
2.7	<i>Stock preservation</i>	40
2.8	<i>Random mutagenesis</i>	40
	<i>(a) Mutagenic polymerase chain reaction (PCR)</i>	
	<i>(b) Propagation in the mutD bacterial strain</i>	
2.9	<i>Rapid isolation of plasmid and chromosomal DNA from yeast</i>	41
2.10	<i>Southern hybridization</i>	41
2.11	<i>DNA sequence analysis</i>	42
2.12	<i>Expression of Protein A fusion proteins from the P_{lac} promoter</i>	42
2.13	<i>Expression of β-galactosidase fusion proteins from the P_R promoter</i>	42

2.14	<i>Fractionation of bacterial cultures by centrifugation</i>	43
2.15	<i>Use of Fast Flow IgG Sepharose for the purification of Protein A fusion proteins</i>	43
2.16	<i>Yeast glass bead homogenate</i>	44
2.17	<i>Fractionation of crude yeast cell homogenate</i>	44
2.18	<i>Chemical mutagenesis of yeast cells using EMS</i>	44
2.19	<i>Construction of yeast strains and random spore analysis</i>	45
2.20	<i>Determination of yeast mating type</i>	45
2.21	<i>Processing of yeast cells for viewing under the Electron Microscope using LR white resin</i>	46
	<i>(a) Fixation of the yeast cells</i>	
	<i>(b) Periodate treatment of fixed yeast cells</i>	
	<i>(c) Dehydration of yeast cells</i>	
	<i>(d) Infiltration of dehydrated yeast cells with LR white resin</i>	
	<i>(e) Embedding of yeast cells in solid LR resin</i>	
	<i>(f) Grid preparation, sectioning and staining</i>	
2.22	<i>Electrophoretic separation and detection of proteins</i>	48
	<i>(a) Coomassie Blue staining</i>	
	<i>(b) Silver staining</i>	
2.23	<i>Semi-dry blotting of proteins</i>	49
2.24	<i>Western blot detection of proteins</i>	50
2.25	<i>Isolation of antigen and the immunization of rabbits for antibody production</i>	50
2.26	<i>Affinity purification of polyclonal antibodies</i>	51
2.27	<i>Detection of β-lactamase activity</i>	52

Chapter 3

<i>Production of a Sec18p-Protein A fusion protein for use in attempts to affinity purify Sec18p-containing protein complexes</i>	53
3.1 <i>Introduction</i>	54
3.2 <i>Construction of a C-terminal tagged version of Sec18 protein</i>	54
3.3 <i>Detection of active fusion protein in vivo</i>	57
3.4 <i>Fractionation studies showing the intracellular distribution of fusion protein</i>	57
3.5 <i>Initial affinity purification studies of the fusion protein using IgG Sepharose</i>	59
3.6 <i>Conclusion</i>	63

Appendix to Chapter 3

<i>Production of affinity purified polyclonal antibodies</i>	76
3.A.1 <i>Production of affinity purified antibodies against Sec18p and Sec17p</i>	77

Chapter 4

<i>Random mutagenesis of the SEC18 gene and isolation of dominant negative alleles of this gene</i>	82
4.1 <i>Introduction</i>	83
4.2 <i>Insertion of the SEC18 gene downstream of the GAL1 promoter within yeast expression vectors</i>	84
4.3 <i>Random mutagenesis of the SEC18 gene and screening for dominant negative mutations</i>	84

	<i>within the SEC18 gene</i>	
4.4	<i>Analysis of mutations within the SEC18 gene</i>	86
4.5	<i>Further analysis of the SEC18-112 mutant allele</i>	87
4.6	<i>Further analysis of the SEC18-109 mutant allele</i>	88
4.7	<i>Conclusion</i>	89
 Chapter 5		
	<i>Characterization of the dominant negative forms of the Sec18p</i>	106
5.1	<i>Introduction</i>	107
5.2	<i>Biochemical analysis</i>	107
5.2.1	<i>Growth curves of the mutants</i>	107
5.2.2	<i>Secretory phenotype of the mutants</i>	107
5.2.3	<i>Density increase of the mutants</i>	109
5.2.4	<i>Internal processing of carboxypeptidase Y (CPY)</i>	110
5.3	<i>Morphological analysis</i>	111
5.3.1	<i>Electron Microscopy: Internal membranes</i>	111
5.4	<i>Conclusion</i>	113
 Chapter 6		
	<i>Isolation of galactose-resistant revertants of strains harbouring dominant negative SEC18 mutations</i>	133
6.1	<i>Introduction</i>	134
6.2	<i>Isolation of suppressors of the DNM forms of the SEC18 gene</i>	134
6.3	<i>Large scale purification and size fractionation of yeast genomic DNA</i>	137

6.4	<i>Attempted construction of a yeast genomic DNA library</i>	138
6.5	<i>Conclusion</i>	139
 Chapter 7		
	<i>Discussion</i>	149
7.1	<i>Discussion</i>	150
 Appendix		
	<i>Bacterial / yeast strains and plasmids used throughout this study</i>	156
	 <i>Bibliography</i>	167

<i>List of figures</i>	<i>Page</i>
<i>Figure 1.1 Processing of α-factor and carboxypeptidase Y through the secretory pathway.</i>	9
<i>Figure 1.2 Two small GTP-binding protein dependent mechanisms within yeast that promote vesicle budding.</i>	31
<i>Figure 1.3 Regulation of vesicle docking/fusion by small GTP binding proteins.</i>	34
<i>Figure 3.1 Insertion of an FspI site into the SEC18 gene by site directed mutagenesis.</i>	64
<i>Figure 3.2 Fractionation profile of Sec18pra fusion protein in the presence / absence of ATP</i>	66
<i>Figure 3.3 Fractionation profile of the Sec18pra fusion protein under various conditions.</i>	68
<i>Figure 3.4 Affinity purification of Sec18pra fusion protein from yeast in the presence of an ATP regenerating system.</i>	70
<i>Figure 3.5 Affinity purification of the Sec18pra fusion protein from yeast in the presence of ATP without any magnesium.</i>	72
<i>Figure 3.6 Analysis of bound material for the presence of wild-type Sec18p and Sec17p.</i>	74
<i>Figure 3.A.1 Detection of Sec18p by affinity purified polyclonal antibodies</i>	78
<i>Figure 3.A.2 Detection of Sec17p by affinity purified polyclonal antibodies</i>	80
<i>Figure 4.1 Cotransformation of mutagenised SEC18 gene and a gapped plasmid into the yeast strain JRY188.</i>	90

Figure 4.2	<i>Screening for a dominant negative Sec18p phenotype in the yeast strain JRY188.</i>	93
Figure 4.3	<i>Revised sequence of the SEC18 gene and predicted amino acid sequence.</i>	95
Figure 4.4	<i>Construction and expression of hybrid genes between the SEC18-112 gene and the wild-type SEC18 gene.</i>	99
Figure 4.5	<i>Sequence alignment between the <i>S. cerevisiae</i> Sec18p and the <i>C. albicans</i> Sec18p and mammalian NSF.</i>	101
Figure 4.6	<i>The predicted secondary structure of the protein encoded by the mutant allele SEC18-109.</i>	103
Figure 5.1	<i>Growth rate of mutants upon shifting to galactose medium.</i>	115
Figure 5.2	<i>Assay of β-lactamase activity secreted from both wild-type and mutant yeast strains.</i>	117
Figure 5.3	<i>Density enrichment of mutant strains grown on galactose medium.</i>	120
Figure 5.4	<i>Fractionation of density gradients.</i>	122
Figure 5.5	<i>Western blot analysis of carboxypeptidase Y (CPY) processing within both wild-type and mutant yeast strains.</i>	124
Figure 5.6	<i>Electron micrographs of wild-type and mutant yeast strains over a 5 hour time course of growth on galactose medium or at 37°C.</i>	126
Figure 5.7	<i>Electron micrographs of wild-type and mutant yeast strains showing their terminal phenotype after 20 hours of growth on galactose medium.</i>	131

Figure 6.1	<i>Integration of the DNM forms of the SEC18 gene into the genome by homologous recombination.</i>	141
Figure 6.2	<i>Southern blot analysis of integrated strains.</i>	143
Figure 6.3	<i>Size fractionation of partially digested yeast genomic DNA.</i>	145
Figure 6.4	<i>Restriction digest analysis of putative library clones.</i>	147

<i>List of Tables</i>	<i>Page</i>
<i>Table 1 Synaptobrevin, syntaxin and rab proteins in the yeast Saccharomyces cerevisiae.</i>	25
<i>Table 3.1 Complementation of the sec18-1 mutation by a Sec18pra fusion protein.</i>	65
<i>Table 4.1 Extent of mutagenic phenotype observed for three random mutagenic strategies.</i>	92
<i>Table 4.2 Sequence analysis of two DNM forms of the SEC18 gene.</i>	98
<i>Table 5.1 Internal and external β-lactamase activity within a Class I and Class II mutant.</i>	119
<i>Table A.1 Bacterial strains used in this study.</i>	157
<i>Table A.2 Yeast strains used in this study.</i>	158
<i>Table A.3 Source of plasmids used in this study.</i>	160
<i>Table A.4 Plasmids constructed in this study.</i>	162

Chapter 1
Introduction

1.1 Introduction

Most proteins in eukaryotic cells are synthesised in the cytosol but their final site of action may be within a number of organelles such as the lysosome (vacuole in yeast cells), the plasma membrane or the external environment (Palade *et al.*, 1975).

Proteins destined for sites other than the cytosol, mitochondria or peroxisome are initially cotranslationally translocated across the endoplasmic reticulum (ER) and are transported through a series of membrane-bound compartments, undergoing successive modifications before they are targeted to their final point of action. How do proteins become targeted to this biosynthetic pathway ?

1.1.1 Targeting of proteins to the endoplasmic reticulum (ER)

Early work identified specific signal sequences at the N-terminus of a number of nascent polypeptide chains and these were found to consist of a very hydrophobic sequence of 15-30 amino acids (von Heijne *et al.*, 1985). For most proteins destined to be secreted from the cell or for the lysosome the signal sequence is cleaved off once the proteins are in the lumen of the ER, whereas in membrane proteins this signal sequence often acts as a membrane anchor and remains intact within the protein.

Mammalian cell-free assay systems which reproduced the targeting and translocation of proteins across the ER membrane led to the isolation of a signal recognition particle (SRP) (Walter and Blobel, 1980) and its receptor (Gilmore *et al.*, 1982a & b). The function of these proteins in the translocation process was shown using cell-free translation systems in which the exact fate of the nascent polypeptides could be followed.

Mammalian SRP (an 11S cytoplasmic ribonucleoprotein containing six polypeptides of 72, 68, 54, 19, 14 and 9kDa together with one 7SL RNA molecule) was purified from canine pancreas by following its translocating-promoting activity in a heterologous *in vitro* system. The particle has been shown to cycle between cytosolic

and membrane-bound forms and is attributed three main functions *in vivo* : (1) the 54kDa subunit binds the signal sequence on nascent polypeptide chains via a methionine-rich (M domain) region in a GTP-dependent manner (Bernstein *et al.*, 1989; High *et al.*, 1991; Zopf *et al.*, 1990). Although the M domain (SRP54M) is sufficient for binding signal sequences in the absence of the GTP binding domain (G domain), chemical modifications of the G domain blocked binding implying that this region was involved in the regulation of the binding of signal sequences (Lutcke *et al.*, 1992). (2) Binding of the SRP to the signal sequence inhibits translation as seen *in vitro* possibly allowing time for the movement and targeting of the ribosomal complex to the ER membrane where (3) the SRP binds to the SRP receptor, a heterodimeric protein (SR α and SR β) found anchored in the ER membrane. The nature of the interaction of the SRP G domain with both SR α and SR β subunits (which both have GTPase activities) is not known but the hydrolysis of GTP may in some way stimulate the dissociation of SRP and release the translational block on the nascent polypeptide (for review see Rapoport, 1991), resulting in translocation of the nascent chain.

1.1.2 Translocation of proteins into the ER

Once targeted to the ER how do proteins cross the membrane bilayer ? Protease protection assays have implicated the presence of proteins in the ER membrane which have been postulated to form a protein 'translocon' that is required for this translocation process across the lipid bilayer (Connolly *et al.*, 1989). Incorporation of photoreactive chemical groups into nascent polypeptide chains has allowed the cross-linking of these polypeptides to neighbouring proteins in the ER membrane bilayer. In this way a number of protein components of the translocon have been identified e.g ribophorins, a 35-39kD protein, translocating chain associated membrane protein (Gorlich *et al.*, 1992) and a 205kD protein with an as yet unknown function (Anderson and Denny, 1992). The actual mechanisms involved in the translocation of

proteins across the ER membrane are still not fully understood.

A number of yeast mutants were isolated which are defective in the translocation of soluble proteins into the ER lumen (*sec61*, 62 and 63) ; (Stirling *et al.*, 1992). The isolation of such mutants has allowed the examination of protein translocation efficiencies *in vivo*. Chemical crosslinking studies have shown that the protein products of these genes interact in a large complex with a further two unidentified proteins (Deshaies *et al.*, 1991). The translocation of soluble proteins such as α -factor precursor or carboxypeptidase Y (CPY) precursor were shown to be blocked in single or double combinations of mutants *sec61*, 62 and 63. However, other proteins such as invertase or the vacuolar membrane protein dipeptidylaminopeptidase B (DPAPB) were affected to a lesser extent. A stricter genetic screen was employed to try and isolate mutants that were defective in the translocation of membrane proteins; a new mutant allele of *sec61* was isolated and a further new gene identified (*SEC65*) the gene product of which is homologous to the 19kDa mammalian SRP subunit (Hann *et al.*, 1992; Stirling and Hewitt, 1992). Yeast homologues of both the 54kDa subunit of SRP (SRP54) and the 7SL RNA (scR1) have been identified (Hann and Walter, 1991) as well as a receptor for SRP (Ogg *et al.*, 1992). Surprisingly, deletion mutants of both the SRP and the SRP receptor have shown that these proteins although important for cell growth and secretion are not essential. In fact mutants that are left long enough undergo physiological changes which allow them to overcome these deficiencies and start to secrete and grow once more.

The translocation efficiency across the ER membrane for a range of proteins was monitored in mutants which had a loss of SRP or SRP receptor function. Surprisingly, the efficiencies of translocation were dependent on the type of protein monitored. Translocation of Kar2p (the yeast homologue of BiP) and DPAPB was severely disrupted whereas translocation of α -factor precursor protein was disrupted

to a lesser extent. The efficiency of CPY translocation into the ER was not affected in these mutants. These observations are of the inverse to those observed in the *sec61*, 62 and 63 secretory mutants although the significance of this relationship is as yet unknown.

However, in both groups of mutants discussed a large variation in translocation phenotype is observed for different proteins which suggests that a variety of translocation pathways exist within eukaryotic cells that allow the insertion of proteins into or through the ER membrane bilayer. The most efficient pathway is the SRP-dependent cotranslational translocation of proteins into the ER (observed for DPAPB and Kar2p) but proteins may be inserted either posttranslationally (possibly in the case of α -factor precursor protein) in an hsp70/chaperone dependent manner or by a cotranslational SRP independent pathway (possibly in the case of CPY protein). Although the alternative pathways could explain the results seen in the mutant yeast cells these may represent minor salvage pathways which are used in stressed situations and may not represent the major route taken for the translocation of proteins within a wild-type yeast cell.

1.1.3 Processing of proteins within the ER

During the translation/translocation process nascent polypeptide chains are acted upon by a number of modifying enzymes resident in the lumen of the ER such as the molecular chaperone BiP (Kar2p in yeast), signal peptidase and a protein disulphide isomerase (for review see Gething and Sambrook, 1992). Nascent polypeptides enter the lumen of the ER in an extended configuration exposing hydrophobic regions that are normally hidden in the folded conformation of a mature protein. The molecular chaperone BiP (an ATPase) is thought to bind these polypeptides to mask/exposed regions and thus prevent protein aggregation and allow correct protein folding to occur. This has been corroborated by crosslinking studies within yeast cells in which

proteins trapped in the process of translocation were found to be crosslinked to Sec61p and BiP/Kar2p (Sanders *et al.*, 1992).

Most secretory proteins are glycoproteins and in yeast are generally mannoproteins containing N- and O-linked carbohydrate moieties. Synthesis of such N-linked glycosylation occurs during protein translocation through the ER in a similar way in both yeast and mammalian cells. A 'core' oligosaccharide (GlcNAc)₂-Man₉-Glc₃ is transferred in a single step from a dolichol-phosphate carrier to Asn residues on the nascent protein to give N-glycosidic linkages. The glucose (Glc₃) residues are then trimmed off before the protein is transported to the Golgi.

1.1.4 Processing of proteins within the Golgi apparatus

In mammalian cells as a glycoprotein is transported through the Golgi apparatus it is processed by a series of mannosidases and sugar transferases to give a final complex structure containing Man, plus additional GlcNAc, galactose, fucose and sialic acid residues. The distribution of the specific enzyme(s) responsible for this series of processing events has been elucidated using immunolocalization and subcellular fractionation techniques and has demonstrated that the mammalian Golgi complex is divided into at least three spatially and functionally distinct regions: *cis* Golgi network (CGN), medial, and *trans* Golgi network (TGN), (for review see Dunphy and Rothman, 1985). These regions can be morphologically defined as distinct stacked Golgi cisternae (for review see Mellman and Simons, 1992).

The compartmental organization of the Golgi complex within yeast cells however has not been extensively characterized. The fission yeast *Schizosaccharomyces pombe* has a Golgi complex morphologically similar to that of mammalian cells (Chappell and Warren, 1989) however, stacked Golgi cisternae are rarely seen within the wild-type budding yeast, *S. cerevisiae*. Stacked and exaggerated Golgi cisternae have been

observed within two temperature sensitive secretory mutant cells (*sec7* and *sec14*) in which there is a block in protein traffic through the Golgi at the restrictive temperature (Esmon *et al.*, 1981; Stevens *et al.*, 1982) however this may not represent the wild-type situation. The characterization of the Golgi complex within *S. cerevisiae* has been achieved by following the processing events of specific glycoproteins (α -factor (α f), carboxypeptidase Y (CPY), invertase and acid phosphatase) within *sec* mutants both at the permissive and restrictive temperature, so defining distinct Golgi compartments (Stevens *et al.*, 1982).

The pro- α f-polypeptide contains four repeated copies of the mature 13 amino acid peptide (Kurjan and Herskowitz, 1982) which are excised from the precursor in the Golgi complex by the concerted action of Kex2 endopeptidase (Kex2p), Kex1 carboxypeptidase and dipeptidylaminopeptidase A (Ste13p) (Bussey, 1988; Fuller *et al.*, 1988). Enzyme(s) that catalyse α 1,6 Man addition have been localised to an early Golgi compartment that lacks α 1,3 and Kex2 protease activities (Franzusoff and Schekman, 1989). In addition membranes enriched for α 1,3 mannosyltransferase and Kex2 protease activities can be partially separated within Percoll gradients (Cunningham and Wickner, 1989).

From such studies three functionally distinct compartments have been defined in the Golgi apparatus of the yeast *S. cerevisiae*. Proteins transported from the ER first pass through a compartment in which the glycoproteins are extended by an α 1,6 mannosyl transferase. This compartment may be the equivalent of the CGN or represent an early compartment of the medial Golgi. In a further medial Golgi compartment the glycoproteins are modified by α 1,2 and α 1,3 mannosyltransferases, after which these proteins are transported to the functional equivalent of the mammalian TGN which contains a set of proteolytic enzymes: Kex2p, Kex1p and DPAP A which act on the α f pheromone (Bryant and Boyd, 1993). This

compartment also seems to represent the site where vacuolar proteins (e.g. CPY) are sorted and directed to their final destinations (Graham and Emr, 1991) (see *Figure 1.1.*).

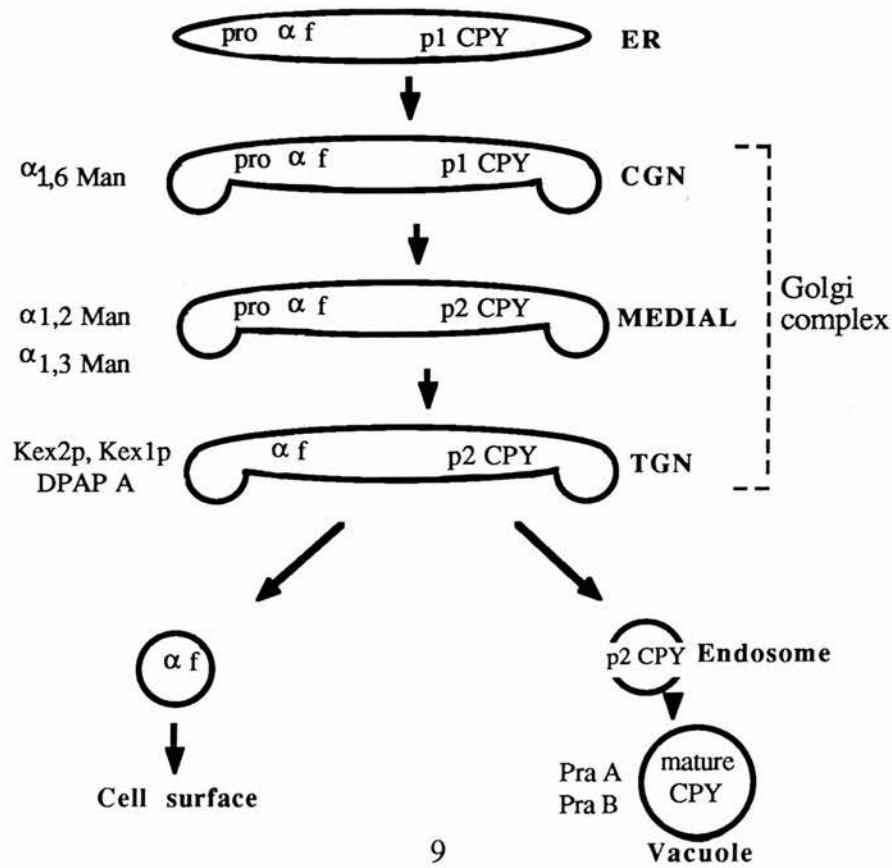
Indirect immunofluorescence staining with antibodies directed against Golgi proteins, such as Kex2p, Kex1p and DPAP A give a pattern of dispersed patches (Redding et al., 1991) and immunoelectron microscopy has shown the presence of disc-like membranes dispersed throughout the cytoplasm (Preuss *et al.*, 1992). Thus, although the yeast and mammalian Golgi apparatus seem to be functionally similar they may not be spatially organised in the same way.

Proteins destined for secretion or for intracellular organelles progress through a number of membrane bound compartments in a constant flow of membrane transport vesicles. How does the cell ensure efficient sorting of exported proteins and resident organellar proteins ? Mutational analysis of resident soluble ER proteins identified a carboxy-terminal tetrapeptide KDEL which was shown to be both necessary and sufficient for the retention of at least one of these proteins (BiP) in the ER (Munro and Pelham, 1987). In the yeast *S. cerevisiae* the carboxy-terminal sequence HDEL has been shown to be sufficient for resident ER protein retention (Pelham *et al.*, 1988). How are these retention signals recognised by the cell ? Studies involving a KDEL-tagged peptide revealed that this peptide was subject to post-ER processing although retained within the ER (Pelham, 1988). This was confirmed in a yeast system by showing that fusion proteins (pro- α -factor and invertase) bearing C-terminal HDEL tags accumulated internally, whereas the corresponding protein which lacked this tag was rapidly processed and secreted from the cell. Fractionation analysis of these cells confirmed that the accumulated protein was in the ER but had been modified by the addition of α 1,6 Man residues, a reaction that is thought to occur in an early Golgi compartment (Kukuruzinska *et al.*, 1987). Retention of such Golgi-modified species

Figure 1.1 Processing of α -factor and carboxypeptidase Y through the secretory pathway

This diagram represents a summary of the processing of two glycoproteins that occurs as they transverse the Golgi apparatus as defined within the *sec18-1* mutant cells (Graham and Emr, 1991). CPY is synthesised as a proenzyme and acquires core oligosaccharides in the ER (the p1 form of CPY). The oligosaccharides are then extended through the medial and TGN (the p2 form of CPY) until it is sorted from the TGN via the endosome to the vacuole where it undergoes proteolytic cleavage by proteinase A and proteinase B (to give the mature form; mCPY).

Pro- α -factor (the precursor form of α -factor) also undergoes core glycosylation and oligosaccharide extensions within the ER and Golgi apparatus respectively. Proteolytic cleavage occurs in the TGN by the concerted action of Kex2p, Kex1p and DPAP A to produce the mature α -factor that is sorted into vesicles and secreted from the cell.



in the ER is dependent on a receptor-mediated recycling event since overexpression of HDEL fusion constructs leads to saturation of the system with concomitant secretion (Dean and Pelham, 1990). This receptor is encoded by the *ERD2* gene in *S. cerevisiae* (Semenza *et al.*, 1990) and the human homologue of this receptor has also been identified (Lewis and Pelham, 1990).

In addition to the recycling of resident proteins to the ER, proteins are held back within the ER until they are in the correct conformation before further transport through the secretory pathway. Mutants of haemagglutinin which block various stages of the protein folding pathway were unable to exit from the ER and were found to be bound to BiP (Gething *et al.*, 1986). It seems that unfolded proteins are retained transiently in the ER by the action of the chaperone BiP which binds to nascent polypeptide chains until the correct folding is observed.

1.1.5 Bulk flow of proteins through the secretory pathway

The presence of these organelle specific retention signals has led to a model in which proteins lacking a specific retention sequence are moved from the ER in the lumen and walls of unselective transport vesicles in a rapid `bulk flow` process and are finally secreted from the cell. Only proteins that contain specific retention signals are held back or targeted to specific organelles e.g the *cis* face of the Golgi is thought to be a salvage compartment and is the main site of recognition and recycling of escaped ER resident proteins. Studies within CHO cells using signal-less tripeptides with acceptor sites for glycosylation showed that these peptides were glycosylated in the ER and secreted efficiently from the cells (Wieland *et al.*, 1987). However, recent analysis within *S. cerevisiae* has shown that peptide export from the ER is not inhibited by antibodies against proteins involved in formation of transport vesicles, the peptides do not reside in membrane bound vesicles after export and they do not receive Golgi specific modifications. This suggests that the exit route for tripeptides is different

from that of secreted proteins (Romisch and Schekman, 1992). Mutational analysis of Golgi integral membrane proteins has shown their mislocalization to the vacuole and not to the plasma membrane suggesting that there could be a different default pathway for membrane proteins within the cell (Cooper and Bussey, 1992; Roberts *et al.*, 1992).

Because so many transport steps require vesicle intermediates the understanding of the common mechanisms underlying vesicle formation, targeting and fusion are very important. A great deal of progress has been made in the past three years in the identification of a number of components that are involved in vesicle fusion events that occur throughout the secretory pathway of both mammalian cells using a variety of *in vitro* transport assays and in the yeast *S. cerevisiae* by the isolation of a number of secretory mutants.

1.1.6 Secretory mutants of Saccharomyces cerevisiae

The yeast *S. cerevisiae* has been shown to have a secretory pathway similar to that seen in mammalian cells which allows the use of a number of genetic techniques to study this process. An initial observation that a mutant yeast strain defective in growth at the restrictive temperature of 37°C had an increased density compared with a wild-type yeast strain on sucrose gradients (Novick and Schekman, 1979) led to a density enrichment screen of chemically mutagenised cells at 37°C to isolate temperature sensitive (ts) secretory (*sec*) mutants. A number of mutants isolated in this way showed a block in export of cell surface-associated proteins (invertase and acid phosphatase) and were classified into three main groups: Class A mutants accumulated invertase internally at the restrictive temperature; Class B mutants had no invertase accumulation although protein synthesis still occurred at the restrictive temperature; and Class C mutants exhibited temperature sensitive protein synthesis.

Morphological analysis of Class A mutants revealed that they accumulated one of three distinct organelle structures depending on the position at which the secretory pathway was blocked e.g. endoplasmic reticulum (ER), Golgi-like structures called Berkeley bodies and 80-100 nm secretory vesicles representing transport intermediates *en route* to the plasma membrane. Genetic analysis of these mutants identified 23 complementation groups (*sec1-23*) acting throughout the secretory pathway indicating that at least 23 independent gene products are involved in the export of proteins from the cell (Novick *et al.*, 1980). Two additional mutants *bet1* and *bet2* (blocked in early transport) were isolated using a [³H] mannose suicide selection in which cells defective in protein transport to the Golgi apparatus do not receive lethal mannose additions to their glycoproteins (Newman and Ferro-Novick, 1987).

Genes which have an effect in independent pathways that contribute to the same process show both the single mutant phenotypes in a double mutant whereas genes that act in the same pathway exhibit the phenotype of the earliest acting mutant in a double mutant. The construction of double *sec* mutants allowed the position at which these genes act within the pathway to be identified thus dissecting the pathway into three main stages (a) Early ER to Golgi transport requiring at least nine *sec* gene products (b) Packaging of proteins from the late Golgi into secretory vesicles in an energy-dependent manner and (d) secretory vesicle to plasma membrane transport (Novick *et al.*, 1981).

Further morphological and genetical analysis has been employed to dissect early ER to Golgi transport (Kaiser and Schekman, 1990). Of the nine *sec* mutants acting at this stage three Class II mutants (*sec 17, 18, and 22*) accumulate an extended ER and 50 nm vesicles. Histochemical staining of a cargo protein within these mutants suggests that the 50 nm vesicles are ER-derived. Formation of such vesicles is not seen in the Class I mutants (*sec 12, 13, 16, and 23*) at the restrictive temperature. Double

mutant analysis of Class I/Class II mutant combinations suggests that the early *sec* mutants can be sub-divided into functions of vesicle formation (Class I mutants) and vesicle fusion (Class II mutants) functions. *sec17,18* double mutants exhibit synthetic lethality (i.e double mutants are lethal at the permissive temperature 24°C) which has been taken to imply an interaction of these gene products at the same biochemical step during intermediate vesicle fusion to the *cis* Golgi apparatus. Since transport from the ER precedes other events in secretion no step after this stage in the exocytotic pathway may be monitored genetically and so these genes may act at multiple stages of the secretory pathway.

Analysis of fluid-phase endocytosis in yeast was carried out by measuring the uptake of a fluorescent dye into the vacuole of the cell and showed that most of the early acting *sec* mutants are not defective in endocytosis at 37°C (Riezman, 1985). However both *sec18* and *sec23* mutant alleles seemed to be defective in endocytosis at the restrictive temperature implying that both of these gene products also act later in the secretory pathway or in the endocytotic pathway. In contrast to these results all the late-acting *sec* mutants were blocked in the endocytotic pathway also at the restrictive temperature. This suggests two possible models of the interaction between the secretory and endocytotic pathways in yeast. Either part of the endocytotic and secretory pathways are coupled therefore a late block in secretion would block endocytosis and an early one would not, or the two pathways are separate but share common gene products.

1.2 Intracellular membrane traffic

1.2.1 Cell free transport systems

The use of an *in vitro* transport system simulating intra-Golgi transport between the *cis* and medial Golgi compartments has led to the identification of proteins involved in vesicle fusion events within a mammalian cell free system (Balch *et al.*, 1984; Braell

et al., 1984). This system relies on the incubation of a 'donor' membrane fraction with a biochemically distinct 'acceptor' fraction and transport is monitored by the transfer of the cargo protein VSV-G (vesicular stomatitis virus G protein) between these fractions. This protein is a viral outer membrane glycoprotein which is modified when in the Golgi compartment by the addition of GlcNAc residues. The two populations of Golgi membranes are isolated from Chinese Hamster Ovary (CHO) cells and distinguished in that the 'donor' population is isolated from mutant 15B-CHO cells infected with VSV-G protein. These mutant cells are defective in the enzyme *N*-acylglucosaminyl transferase I (normally resident in the medial Golgi) and so cannot transfer an *N*-acylglucosamine group from UDP-GlcNAc to the cargo VSV-G protein. The acceptor population is prepared from wild-type CHO cell population not infected with VSV-G protein. Co-incubation of these two membrane preparations results in the transfer of VSV-G protein from the donor to the acceptor compartment where it will receive a [³H]-UDP-GlcNAc moiety due to the presence of GlcNAc transferase I in the wild-type acceptor population. The amount of label found in the acceptor population is therefore a measure of the vesicular transport that has occurred between these two compartments. Using this system it was found that there were a number of requirements for transport, such as the presence of an ATP regenerating system, fatty acyl CoA (Pfanner *et al.*, 1990), physiological conditions of temperature and pH, and soluble proteins present in a high speed supernatant fraction obtained from CHO cells (termed cytosol). Cytosol fractions prepared from other sources such as bovine brain, and wild-type yeast cells could also substitute for CHO cell cytosol.

Inter-cisternal Golgi transport using this *in vitro* system could be sub-divided into a number of intermediary stages using a variety of inhibitors (Orci *et al.*, 1989). The stages of vesicular transport can be divided into the initial budding of vesicles from the donor membrane to produce transport intermediates which are surrounded by a non-clathrin coat and accumulate *in vitro* when transport is arrested using GTP γ S (a non-

hydrolysable analogue of GTP) or AlF_4^- . These coated vesicles are then targeted to the acceptor membrane where they attach and are uncoated ready for bilayer fusion to occur. Transport is inhibited by incubating the membranes on ice with a low concentration (1 mM) of the sulphhydryl reagent *N*-ethylmaleimide (NEM). This substance arrests transport producing vesicles attached to acceptor membranes in an uncoated state ready for fusion .

The use of two stage incubations within this cell-free assay system revealed three intermediate stages in vesicular transport that seemed to correspond to the initial budding of transport vesicles from the donor membrane and subsequent maturation of that intermediate vesicle through a prefusion complex leading to final membrane fusion (Balch *et al.*, 1984; Wattenberg *et al.*, 1986). In the first stage, the donor membrane is 'primed' and buds off to form an early primed donor intermediate in the absence of acceptor Golgi membranes. This stage requires the presence of ATP and cytosol and is sensitive to NEM. The second stage involves the production of a prefusion intermediate that is dependent on the presence of the acceptor membrane and therefore represents the attachment of the 'primed' donor vesicle to this membrane. This low cytosol requiring intermediate (LCRI) was identified by the requirement of a high concentration of cytosol for its formation whereas its consumption is less dependent on cytosol. Subsequent maturation of this LCRI occurs in an ATP and cytosol dependent manner to produce an NEM- resistant prefusion intermediate and then fusion.

Preliminary fractionation studies of bovine brain cytosol revealed that a distinct cytosolic component was required to consume this LCRI complex. This protein (Factor B) has an apparent molecular mass of 25 kDa and is inactive in the priming stage of vesicular transport (Wattenberg and Rothman, 1986). A similar protein of 25kDa was isolated from the yeast *S. cerevisiae* and termed protein operating in

prefusion (POP).

Molecular cloning of POP revealed it to encode uridine monophosphokinase (UMPkinase). The cell-free assay system measuring protein transport between compartments of the Golgi follows the glycosylation of a glycoprotein, G protein (as described earlier). Glycosylation within this system was assumed to occur immediately on fusion of the vesicles and therefore this model takes the kinetics of glycosylation to reflect those of vesicle fusion. However, Golgi uptake of UDP-GlcNAc was shown to be enhanced in the presence of UMPkinase, leading to an increased glycosylation signal and thus a false assessment of vesicle fusion. Reinterpretation of the data revealed that the original kinetic analysis of vesicle traffic within this system was not correct and the LCRI did in fact represent the vesicle fusion event (Hiebsch and Wattenberg, 1992). This study highlighted the limitations that are imposed upon a system when using *in vitro* analysis.

1.2.2 NEM sensitive factor (NSF)

An NEM sensitive factor (NSF) was identified within CHO cell cytosol by its ability to restore intracisternal transport within an NEM-inhibited *in vitro* transport system and was purified from this cytosol in the presence of ATP (Block *et al.*, 1988). The purified protein was partially sequenced and this sequence was used to create degenerate oligonucleotides to clone the full length cDNA sequence. NSF is a homotetramer consisting of 76kDa subunits and is a soluble protein which peripherally associates with membranes (Wilson *et al.*, 1989). At which point in vesicle transport does NSF act ?

Using the previously defined *in vitro* assay system for vesicular transport a number of preincubation experiments revealed that NSF was required for vesicle fusion rather than budding (Malhotra *et al.*, 1988). In the absence of NSF nearly twice the number

of intermediate vesicles accumulate during cell-free preparations and under the electron microscope these vesicles are seen to be uncoated suggesting that they have already uncoated and docked on the acceptor membrane before NSF is required. Specifically it was shown that NSF is required to form the prefusion LCRI complex in the presence of ATP. From this it seems that NSF is not an actual 'fusogen' but is involved with a number of other cytosolic (such as Factor B) and membrane factors to form a multimeric complex that promotes bilayer fusion.

NSF activity is stabilised in *in vitro* transport assays by the presence of ATP, and it has been shown that NSF contains two distinct but homologous ATP-binding domains and has a low ATPase activity (Wilson *et al.*, 1989; Tagaya *et al.*, 1993) which although lower than other ATPases, (eg, it is about 50 fold lower than that of chromaffin granule ATPase) is comparable to that of p97 (Peters *et al.*, 1990). Both NSF and p97 have been proposed to be members of a novel ATPase family (Erdmann *et al.*, 1991). A semi-intact CHO cell system prepared from VSV-infected CHO cells which reconstitutes ER to *cis* Golgi transport has shown that NSF is also required at this stage for fusion of the intermediate vesicle with a target membrane to occur (Beckers *et al.*, 1989). This transport was inhibited both by NEM and a monoclonal antibody against NSF. Is NSF also implicated in the endocytotic pathway of eukaryotic cells? Endocytosis of externally located ligands may be monitored using two synthetic substances that when internalized and delivered to the same cellular compartment form a measurable complex. Fusion of early endocytotic vesicles were shown in this way to require NSF as an essential component (Diaz *et al.*, 1989).

Although most of these kinetic studies suggest NSF is involved in the formation of a prefusion complex once vesicle attachment has occurred, studies using the antimalarial drug primaquine, an inhibitor of bud emergence, in a modified *in vitro* Golgi transport systems has indicated an earlier role for NSF during the formation of functionally

active intermediate transport vesicles (Wattenburg *et al.*, 1992).

The ubiquitous nature of NSF within both the secretory and endocytotic transport pathways seems to suggest that NSF is predominantly involved in a general mechanism that allows fusion of membrane bilayers to occur. It seems unlikely that NSF is involved in the specificity observed in vesicle fusions; presumably other proteins may confer such a role. Reassociation of purified NSF with treated Golgi membranes *in vitro* was shown to require an integral membrane receptor and a crude cytosol fraction containing an NSF attachment protein(s) (Weidman *et al.*, 1989). Binding of NSF is saturable both with respect to the integral membrane protein and to the component(s) of the cytosol fraction.

1.2.3 Soluble NSF attachment proteins (SNAP)

In vitro transport between Golgi membranes is arrested when the Golgi membranes are pretreated with KCl (KGolgi) which removes all peripherally-associated proteins. Transport was restored by a bovine brain cytosol preparation. This cytosol was resolved into three separate pools by gel permeation chromatography (a) NSF fraction (b) Fr 1 and (c) Fr 2. Fr 2 contained three proteins of related size termed α (35kDa), β (36kDa), and γ (39kDa) soluble NSF attachment proteins (SNAP) from their ability to bind NSF to Golgi membranes (Clary *et al.*, 1990).

Individual SNAPs were purified from the Fr2 fraction of bovine brain cytosol and it was shown that each SNAP in addition to having a transport activity could bind NSF independently to the Golgi membrane. SNAP activity was shown to be needed at a similar stage in vesicle transport as NSF. Does each SNAP interact differently with NSF in binding to Golgi membranes? *In vitro* binding studies have revealed that the SNAP/NSF interaction does not occur in solution but only in the presence of Golgi membranes (or an artificial solid support). Both α/β and γ SNAP bind to Golgi

membranes at distinct binding sites which are integral membrane proteins that are either within the same polypeptide chain or are separate proteins that are within a tight complex. NSF will only bind Golgi membranes which have already been 'primed' by SNAP binding. Although only one SNAP is sufficient for NSF binding, when both binding sites are filled NSF binds and forms a stable NSF-SNAP complex which may then mediate vesicle fusions. Initial crosslinking studies have revealed an α -SNAP receptor (30-40 kDa) within Golgi membranes and a physical interaction between NSF and α -SNAP has been demonstrated by such crosslinking studies (Whiteheart *et al.*, 1992). The binding of both α -SNAP and γ -SNAP to Golgi membranes was not affected by Mg^{++} /ATP within these studies but NSF has been shown to dissociate from Golgi membranes on ATP hydrolysis which suggests that the NSF-SNAP interaction is being affected rather than the initial SNAP binding.

Recent cloning and sequencing of the cDNAs for α , β and γ SNAP has allowed the expression of recombinant proteins for use in cell free transport assays. α and γ SNAP were found to act synergistically in such intra-Golgi transport systems when the amount of NSF was in excess. Crosslinking studies have shown a direct interaction between α and γ SNAP and both are in contact with NSF and although γ SNAP is not essential for cell-free transport it is thought to stabilise interactions within the fusion complex. β SNAP seems to be a brain-specific isoform possibly specialised in certain types of neurosecretion events (Whiteheart *et al.*, 1993).

1.2.4 Evolutionary conservation

Isolation and characterization of proteins involved in mammalian vesicle fusion has led to the identification of homologous proteins within yeast previously defined by temperature sensitive *sec* mutants which show a block in secretion at the nonpermissive temperature of 37°C. The amino acid sequence of NSF and Sec18p are 48% identical and they both contain two predicted ATP binding sites. However,

in vitro translation studies of the *SEC18* gene revealed two gene products of 82kDa and 84 kDa in contrast to the single 76kDa NSF protein within mammalian cells. The two isoforms of Sec18p may have different functional roles *in vivo* but their significance awaits further mutational analysis to test their individual function within cell-free assay systems. Biochemically, wild-type yeast cytosol has been shown to have NSF activity, by its ability to restore transport to NEM-treated membranes in a CHO cell *in vitro* transport system. This NSF activity in yeast cytosol is inactivated in the absence of ATP and is NEM sensitive (Wilson *et al.*, 1989). The *SEC18* gene was originally isolated by its ability to complement a *sec18-1* ts mutation that blocked transport of proteins between the ER and Golgi apparatus (Eakle *et al.*, 1988). Yeast cytosol from such a mutant grown at either the permissive temperature of 25°C or the nonpermissive temperature of 37°C does not contain NSF activity, directly implicating the Sec18p as the yeast homologue of NSF to be involved in vesicle fusion events. However, experiments using a broken yeast cell assay system which measures transport from the ER to the *cis* Golgi supports the role of Sec18p in a vesicle attachment event (Rexach and Schekman, 1991). On comparison of the primary amino acid sequences both NSF and the *SEC18* gene product have shown homologies to a family of ATPase proteins. This family encompasses many diverse cellular proteins such as the yeast cell cycle protein Cdc48p, the Pas1p protein involved in peroxisome biogenesis and p97 from *Xenopus laevis* oocytes.(Frohlich *et al.*, 1991; Erdmann *et al.*, 1991; Peters *et al.*, 1990).

It has been observed that another yeast mutant, the *sec17-1* ts mutant, also blocked in ER to Golgi transport *in vivo* at the nonpermissive temperature, and that a *sec18-1/sec17-1* double mutant showed synthetic lethality. Cytosol prepared from a *sec17-1* mutant is deficient in SNAP activity, shown by its arresting the *in vitro* transport system and this cytosol can be complemented by the addition of pure α SNAP but not β or γ SNAP activities. Sec17p has been shown to be functionally

equivalent to α SNAP and to interact with Sec18p in binding Golgi membranes in a similar way to NSF/SNAP interactions (Griff *et al.*, 1991). However to date a γ SNAP homologue has not been identified within yeast cells. Not only are NSF/SNAP involved as a complex mediating vesicle fusion in the mammalian system but this mechanism seems to be conserved in evolution between diverse systems. Obviously such general protein recruitment onto membranes requires a membrane receptor which has been shown to interact in a saturable manner with the fusogenic complex.

1.2.5. 20S particle involved in membrane docking/fusion

Coimmunoprecipitation of a recombinant myc tagged NSF (NSFmyc) and α SNAP was shown to be dependent on the presence of an integral membrane receptor protein . This NSF/SNAP receptor was shown to complex with NSF and SNAP during sucrose gradient velocity centrifugation with a sedimentation coefficient characteristic of a 20S complex. This complex could not assemble in the presence of NEM-treated NSFmyc and the complex was also shown to disperse upon ATP hydrolysis (Wilson *et al.*, 1992). The real breakthrough in identifying proteins that interact with both NSF and SNAP came when an affinity purification technique was employed to isolate potential SNAP receptors from the 20S complex within bovine brain cytosol exploiting the stability of the complex in the presence of either Mg-ATP γ S or of ATP without magnesium present (Sollner *et al.*, 1993a). NSFmyc, SNAP, ATP γ S and a detergent- solubilised membrane preparation derived from bovine brain were incubated and complex isolation was achieved using an anti-myc epitope antibody bound to a solid support. The complex was disassembled in the presence of ATP-Mg $^{++}$ and three new proteins were visualised in the eluate along with NSF and SNAP. Each protein was identified by creating tryptic peptides which were then sequenced and found to be derived from proteins that had previously been identified as abundant synaptic proteins: two isoforms of syntaxin (A and B) (Bennet *et al.*, 1992);

synaptobrevin (or VAMP 2) (Baumert *et al.*, 1989; Elferink *et al.*, 1988); and a synaptosomal-associated protein of 25kDa (SNAP 25). Synaptobrevin is an 18kDa protein anchored to the cytoplasmic surface of synaptic vesicles by a carboxy-terminal transmembrane domain whereas syntaxin is a 35kDa protein with a carboxy terminal membrane anchor associated with the presynaptic membrane.

Neurotransmitter release is blocked by a group of structurally related toxin proteins produced by *Clostridium botulinum*. Tetanus toxin and botulinum toxins (A-G) have been shown to be zinc proteases but their mechanism of action seen by the ultimate inhibition of neurotransmitter release has only recently been identified. Using highly purified synaptic vesicles from rat brain it was shown that both tetanus toxin and botulinum toxin B (BoNT/B) selectively cleaves VAMP/synaptobrevin (Schiavo *et al.*, 1993). In another study using intact nerve terminals (synaptosomes) it was shown that BoNT/A selectively cleaves SNAP 25 (Blasi *et al.*, 1993). It also seems likely that BoNT/C will be shown to cleave syntaxin, the third component of the putative docking/fusion complex (Huttner, 1993).

Since each toxin is capable of blocking neurotransmitter release this gives direct evidence that synaptobrevin, SNAP 25 and syntaxin are components of a prefusion or fusion complex in neurons. Since NSF/SNAP seem to be involved as a general fusogenic complex these membrane proteins could associate and mediate a synaptic vesicle fusion event. The specificity of such fusions may be mediated by the interaction of the vesicle SNAP receptor (v-SNARE), synaptobrevin and the target SNAP receptor (t-SNARE), syntaxin in the presynaptic membrane. Within the 20S complex the NSF/SNAP particle may act as a proof-reading mechanism for the interaction between the v- and t-SNAREs to elicit correct vesicle fusion events. The third SNAP receptor isolated, SNAP 25, is a soluble protein which associates with membranes by the addition of palmitoyl moieties to cysteine residues within its

sequence (Oyler *et al.*, 1989). Given the requirement for fatty acyl CoA in vesicular transport systems (Glick and Rothman, 1987) *in vitro* SNAP 25 could in fact aid the membrane association/assembly of the 20S complex and facilitate membrane fusion.

Fusion between cellular membranes can occur constitutively, as seen when transport vesicles from the ER fuse with the Golgi or can be closely regulated, as in secretory storage vesicles fuse with the surface membrane upon receipt of a signal (e.g GTP or calcium). The calcium-regulated protein synaptotagmin (p65) coimmunoprecipitates with syntaxin (Bennet *et al.*, 1992) but it was not affinity purified from bovine brain cytosol in the 20S complex using NSFmyc and so was not thought to be involved in vesicle docking/fusion events. However, recent studies looking at the components within the 20S complex *in vitro* have given some evidence as to the involvement of synaptotagmin in the 20S complex and to the mechanisms involved in protein complex association and dissociation during vesicle docking and fusion events (Sollner *et al.*, 1993b). The three SNAREs (synaptobrevin, syntaxin, and SNAP 25) can form a stable complex in the absence of NSF and α SNAP, thus promoting the idea that the specificity seen during vesicle docking events is through v-SNARE/t-SNARE recognition. Immunolocalization of SNAP 25 and syntaxin to the plasma membrane in PC12 cells seems to suggest that in the case of the synapse the active t-SNARE component may have two subunits.

Synaptotagmin was shown to bind this SNARE complex in the absence of NSF and α SNAP but could be displaced by the addition of pure α SNAP. This may account for the previous absence of synaptotagmin within isolated 20S complex and suggests a common site of binding between α SNAP and synaptotagmin on the SNARE complex. Based on these *in vitro* studies it has been suggested that synaptotagmin acts as a 'fusion clamp' to prevent constitutive fusion from occurring in the absence of a signal for exocytosis (Bennett and Scheller, 1993). Once this signal is received the

synaptotagmin can be displaced by α SNAP binding to the SNARE complex which promotes NSF binding and ultimately leading to bilayer fusion. Upon fusion ATP hydrolysis may cause the release of NSF/SNAP so disrupting the SNARE complex. This specific coupling of membranes and the assembly of fusion complex may be additionally regulated by the interaction of Rab proteins (small GTP binding proteins).

1.2.6 Vesicle SNAP receptors (v-SNAREs) in yeast

The biochemical isolation of these fusion components which act at the synaptic cleft during neurotransmitter release has allowed the identification of a number of homologous proteins in other diverse biological systems. v-SNAREs and t-SNAREs have been previously characterised by genetical analysis within yeast, and have been shown to block/affect secretion at a number of stages (*see Table 1*).

Mutational analysis in *S. cerevisiae* originally identified nine *sec* mutants and two *bet* mutants that participated in ER to Golgi transport (Novick *et al.*, 1981; Newman and Ferro-Novick, 1987). The *SEC22* gene encodes a small integral membrane protein showing homology to mammalian synaptobrevin. The *sec22* mutant allele has been shown to cause a block in secretion at an early stage of the secretory pathway between the ER and Golgi leading to the accumulation of 50 nm vesicles which can be visualised under the EM at the nonpermissive temperature. Genetic analysis of *sec* mutants acting between the ER and Golgi has implicated the *SEC22* gene product in vesicle fusion events at the same stage as the *SEC18* and *SEC17* gene products.

The *ras*-related small GTP binding protein Ypt1p has been proposed to participate in ER to Golgi membrane fusion acting in the regulation through the binding and hydrolysis of GTP (Segev *et al.*, 1988). Multi-copy suppressors of *ypt 1* mutant cells *SLY12* (identical to *BET1*) and *SLY2* (identical to *SEC22*) were identified and their gene products were shown to interact with Ypt1p (Dascher *et al.*, 1991; Ossig *et al.*,

1991; Newman *et al.*, 1992). The primary structures of Sly2p(Sec22p) and Sly12p(Bet1p) resemble that of Vamp1p and synaptobrevin, with the highest region of conservation between the sequences of these proteins being in a short region preceding the membrane spanning domain. *BOS1*, a gene that was originally identified as a multi-copy suppressor of a *bet1* mutation encodes an integral membrane protein required for ER to Golgi transport. Transport-competent vesicle intermediates were isolated from an *in vitro* system reconstituting ER to Golgi transport in permeabilised yeast cells using an antibody against Bos1p.

Table 1 *Synaptobrevin, syntaxin and rab proteins in the yeast Saccharomyces cerevisiae*

Family	Yeast Homologue	Transport step
rab	Ypt1	ER to Golgi
	Sec4	Golgi to PM
	Bet1/Sly12	ER to Golgi
VAMP	Sec22/Sly2	ER to Golgi
	Snc1/Snc2	Golgi to PM
	Sed5	ER to Golgi
SYNTAXIN	Pep12	Golgi to vacuole
	Sso1/Sso2	Golgi to PM

These vesicles were shown to contain Bos1p/Sec22p/and Ypt1p but not Bet1p. This shows both a genetical and biochemical interaction of a v-SNARE (Sly2p/Sec22p) in fusion competent transport intermediates with a small GTP binding protein (Ypt1p). Bet1p is thought to be associated with a denser group of ER membrane vesicles (Lian and Ferro-Novick, 1993).

The two *SNC* genes of *S. cerevisiae* encode protein homologues of synaptobrevin. Haploid mutants of either of these genes show no apparent phenotype but *snc1/snc2* mutants show conditional lethality and accumulate post Golgi transport vesicles similar to those observed at the nonpermissive temperature in a *sec6* mutant in yeast (Protopopov *et al.*, 1993).

1.2.7 Target vesicle SNAP receptors (t-SNAREs) in yeast

Three genes have been identified in *S. cerevisiae* that encode proteins with a carboxy-terminal membrane anchor and display significant homology to syntaxin primarily over a 70 amino acid segment near the membrane anchor region. The first of these proteins is encoded by the *SED5* gene. The *SED5* gene was identified as a multi-copy suppressor of the *erd2* mutation and encodes a 39kDa integral membrane protein which has been localised using immunofluorescence to punctate structures throughout the cell (Hardwick *et al.*, 1992; Hardwick and Pelham, 1992). Another syntaxin-like protein has been identified at a later stage of the secretory pathway encoded by the *PEP12* gene and is involved in the targeting of proteins from the late Golgi compartment to the vacuole (Becherer and Jones, 1992). The two yeast genes *SSO1* and *SSO2* have recently been identified by their ability to suppress in high copy the late acting *sec1-1* secretory mutant and show the closest homology to the mammalian syntaxins (Aalto *et al.*, 1993). A number of other late-acting sec mutants are also suppressed by *SSO1* and *SSO2* genes (*sec 3, 5, 9* and *15*) suggesting the interaction of these syntaxin-like proteins within a membrane associated protein complex. That

this complex interaction is involved in vesicle membrane docking and fusion events is suggested by the accumulation of 100 nm vesicles in the absence of both Sso1p and Sso2p which is characteristic of the phenotype observed for the late-acting *sec* mutants seen at the restrictive temperature. Each stage of vesicle-mediated protein transport seems to have a specific t-SNARE present which may mediate the docking/fusion of specific vesicles to the appropriate target membrane.

What has emerged from both a biochemical and genetical analysis of vesicle membrane fusion events is that there is an assembly of a large multimeric protein complex between two membrane bilayers that facilitates their fusion. Why do two opposing bilayers require such a complex for fusion to happen ? The physical environment surrounding membranes *in vivo* must be taken into consideration. In their natural state membrane bilayers are surrounded by a shell of water molecules bound to the polar head groups that set up a net repulsive force between closely opposed bilayers stopping spontaneous fusion. It seems counterintuitive that a large and bulky protein complex which itself is probably hydrated could facilitate such fusion events. It seems likely that this large complex may have a number of important roles (a) bringing two specific membranes close enough for fusion to occur (b) intricate conformational changes within the protein complex can expose internally orientated hydrophobic residues that may allow local bilayer protrusions to fuse and (c) regulating the number and time allowed for these fusions to occur.

1.3 Regulation of intracellular membrane traffic

1.3.1 GTP binding proteins

A large number of GTP binding proteins have been identified in higher and lower eukaryotes and have been classified into two broad families: the *ras* -like small monomeric GTP binding proteins (20-30 kDa) and the heterotrimeric G proteins ($G_{\alpha\beta\gamma}$). GTP binding proteins are involved both in the response of cells to an

external stimulus which is transduced across the plasma membrane (heterotrimeric G proteins) and in mediating vectorial transport processes such as protein synthesis. By alternating between two different conformations in a nucleotide-dependent manner GTP binding proteins act as molecular switches. GTP binding proteins are active in the GTP bound form and inactive on hydrolysis (not dissociation) of GTP to GDP. This switch can be used to introduce vectoriality into a cellular process, as seen for protein synthesis, via the regulation of GTP-GDP exchange and GTP hydrolysis. Do small GTP binding proteins have a role in such intracellular trafficking processes?

Three lines of evidence support the involvement of small GTP binding proteins in vesicular transport. Firstly, mutations in each of the four yeast genes encoding small GTP binding proteins (*ARF1*, *SAR1*, *YPT1* and *SEC4*) result in defects at various stages of the secretory pathway. The use of *in vitro* transport assays has further subdivided these small GTP binding proteins into two distinct groups; the Sec4/Ypt1 family of proteins which seem to be involved in regulating the docking/fusion of intermediate transport vesicles at the plasma membrane and between the ER and Golgi apparatus respectively, and a subfamily of proteins that are the products of the *SAR1* and *ARF1* genes that appear to be involved in vesicle formation from the ER and vesicle coat formation in the Golgi apparatus respectively. Secondly, the mammalian Rab proteins share 25-60% homology to the Sec4/Ypt1 family of proteins. Over 30 individual Rab proteins have been identified so far, and have been localized to distinct intracellular compartments. A combination of both *in vivo* and *in vitro* studies has shown their probable role in the regulation of both exocytotic and endocytotic transport processes (for review see Zerial and Stenmark, 1993). Finally, the most compelling evidence for the participation of small GTP-binding proteins in vesicular transport is that most *in vitro* transport assay systems are inhibited by the non-hydrolysable analogue of GTP, GTP γ S (although the underlying mechanism may not be exactly identical due to the varying concentration of GTP γ S and cytosol

requirement needed in each case).

1.3.2 Regulation of vesicle formation events

The small GTP-binding proteins Arf1p and Sar1p seem to be implicated in vesicle formation from the Golgi cisternae in mammalian cells and the ER in yeast cells respectively. The Sar1p in yeast is a small GTP-binding protein originally identified as a suppressor of the *sec12-1* mutation and shown to be required for ER to Golgi transport (Nakano *et al.*, 1989). Sar1p is 35% identical to mammalian ARF. The *SAR1* gene however cannot suppress a *sec12* deletion mutant which indicated a need for a direct functional interaction of the Sar1p with the integral membrane glycoprotein Sec12p *in vivo*. *In vitro* studies have also shown that vesicle formation requires the direct interaction of both these proteins (Oka *et al.*, 1991). It was subsequently found that Sec12p was a GDP/GTP exchange factor (Barlowe and Schekman, 1993). Other *SEC* genes have been shown to participate in ER to Golgi transport. The Sec23p was identified as a GTPase activating protein specific for Sar1p and forms a complex with Sec24p (Yoshihisa *et al.*, 1993). A basic model is emerging in which Sar1p with Sec12p binds GTP, associates with the ER and then hydrolyses GTP with the aid of a Sec23p/Sec24p complex to catalyse the formation of transport vesicles (**Figure 1.2**).

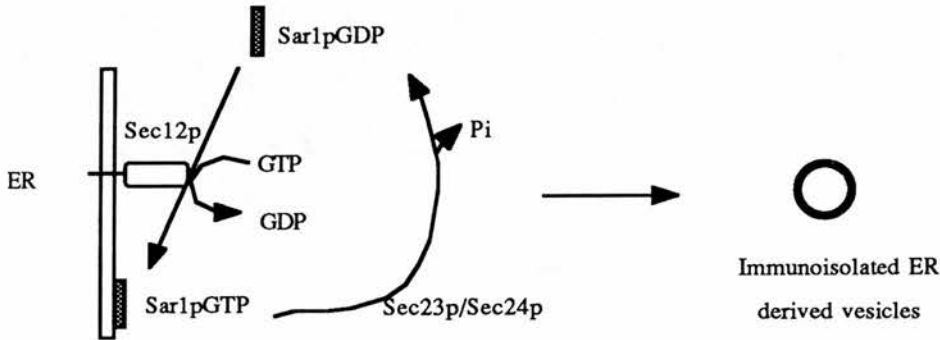
ARF was originally identified as a cofactor involved in the ADP-ribosylation of the α subunit of a trimeric G_s protein by cholera toxin (Schleiffer *et al.*, 1982; Kahn and Gilman, 1984). Mammalian Arfp has been localised to the Golgi apparatus using specific antibodies and is part of the non-clathrin coat surrounding intermediate Golgi vesicles (Stearns *et al.*, 1990; Serafini *et al.*, 1991). Inhibition of intra-Golgi transport assays with GTP γ S results in the accumulation of non-clathrin coated vesicle intermediates and several of the coat proteins (COP) have been characterized: α COP, 160kDa; β COP, 110kDa; β' COP, 102kDa; γ COP, 98kDa; δ COP, 61kDa; ϵ COP,

36kDa and ξ COP, 20kDa and the G protein ARF (Stenbeck *et al.*, 1993; Waters *et al* 1991). Inhibition of transport by NEM however leads to the accumulation of uncoated vesicles (75 nm) which are membrane associated. Coated vesicles seem to be precursors of the uncoated vesicles as inhibition with both GTP γ S and NEM results in the formation of coated vesicles. A cytosolic complex termed 'coatamer' containing the COP subunits of the non-clathrin coated Golgi transport vesicles was purified from bovine brain cytosol. ARF was not associated with this cytosolic coatamer complex yet is part of the non-clathrin coated Golgi vesicles.

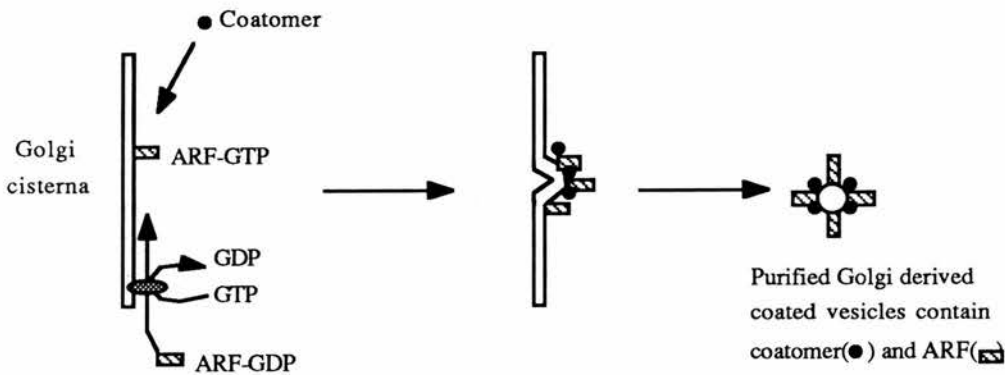
Could ARF be responsible for coat assembly/disassembly at the Golgi membrane and thus mediate vesicle formation? The ability of purified pools of recombinant myristoylated ARF and coatamer to bind to Golgi membranes in the presence of GTP γ S, Brefeldin A (BFA) and AlF_4^- has been analysed. Binding of the coatamer to Golgi membranes was dependent on the presence of ARF and enhanced by both AlF_4^- and GTP γ S but inhibited by BFA (Helms *et al.*, 1993; Donaldson *et al.*, 1991). In this way coatamer assembly on Golgi membranes is thought to involve firstly the activation of ARF by the binding of GTP which allows association with a Golgi membrane receptor in a myristoyl dependent process and activation of coatamer assembly onto Golgi membranes. The interaction of a nucleotide exchange protein on the target membrane could result in GTP hydrolysis and so the [GDP] ARF would then dissociate and disperse coatamer into a cytosolic pool ready for the next round of vesicle transport. BFA inhibits this pathway by inhibiting the exchange of GTP for GDP bound to ARF so that ARF is unable to associate with the Golgi membranes so deterring coatamer assembly on Golgi membranes and preventing vesicle formation (*Figure 1.2*).

Figure 1.2 Two small GTP-binding protein dependent mechanisms within yeast that promote vesicle budding

(a) Sar1p-dependent ER to Golgi transport



(b) Coatomer-dependent intra-cisternal transport.



However, recent data has suggested that there may be a requirement for coatomers during ER to Golgi transport. The *SEC21* gene is required for ER to Golgi transport in *S. cerevisiae* and encodes a protein which participates in a cytosolic complex which has been shown to be the yeast homologue of the mammalian coatomer (Hosobuchi *et al.*, 1992). Sec21p (a 105 kDa protein) is homologous to γ -COP, a coat subunit of non-clathrin coated vesicles (Stenbeck *et al.*, 1992). The functional

role of coatomer in ER to Golgi transport is still to be clarified.

1.3.3 Regulation of intermediate vesicle docking/fusion events

It seems that vesicle docking/fusion within eukaryotic cells involves the initial recognition of a vesicle membrane protein (v-SNARE) to a corresponding target membrane protein (t-SNARE) and the subsequent recruitment of soluble proteins (NSF and SNAPs) to facilitate bilayer fusion (as mentioned in **Section 1.2.5**). One possible method of regulation of this process could be through small GTP-binding proteins at various stages of the secretory pathway (for review see Novick and Brennwald, 1993).

The *SEC4* gene product (Salminen and Novick, 1987) was first implicated in constitutive secretion by the isolation of a *sec4-8* temperature sensitive mutant which blocked secretion late in the pathway accumulating post-Golgi vesicles (80-100 nm) at the restrictive temperature. Sec4p has been localised to the cytoplasmic face of both secretory vesicles and the plasma membrane using polyclonal antibodies. Pulse chase studies however show an initial soluble pool of Sec4p which rapidly becomes membrane-associated in a saturable manner (Goud *et al.*, 1988) suggesting a cyclical process in which Sec4p exchanges between a membrane bound and a soluble form. A genetic interaction has been shown between the *SEC4* gene and several other late acting *SEC* genes (*SEC2*, *5*, *8*, *10* and *15*) and a physical interaction has been shown with Sec8p and Sec15p within a 19.5S particle (Bowser *et al.*, 1992). It seems that the small GTP-binding protein, Sec4p, interacts in some late-acting protein complex possibly involved in the regulation of docking/fusion of secretory vesicles with the plasma membrane in yeast.

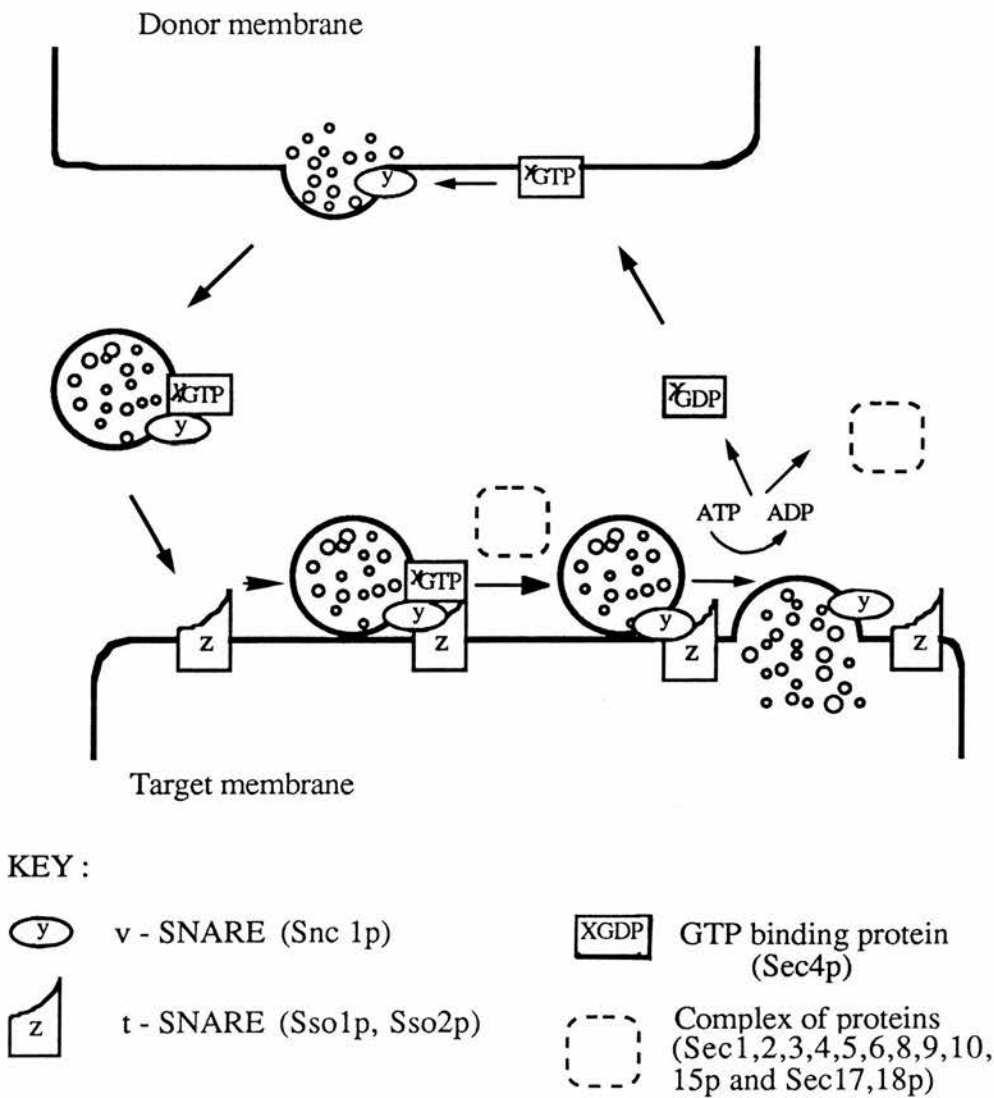
Is there evidence of a synaptobrevin/syntaxin-like interaction involved at this stage in exocytosis? As mentioned before (see **Table 1**) Sec4p and Snpcp have been

colocalized to post-Golgi vesicles and Rab3 (Touchot *et al.*, 1987; Matsui *et al.*, 1988) and VAMP on synaptic vesicles. In both cases a small GTP-binding protein may interact either directly or indirectly with a VAMP-like protein on the surface of a vesicular transport intermediate involved in vesicular docking/fusion events. What of the target membrane? The secretory mutant protein Sec1p was originally identified to be a late acting protein in the exocytotic process. Genetic interaction has been shown between *SEC1* gene and four other genes (*SEC3*, *5*, *9* and *15*), again suggesting a late-acting protein complex which is involved in exocytosis from the plasma membrane. Two yeast genes, *SS01* and *SS02*, were recently identified as suppressors of a *sec1-1* mutation (see **Section 1.2.7**) and have homology to syntaxins.

These studies suggest a model in which a v-SNARE (Snc protein) interacts with a partner t-SNARE (Sso1p or Sso2p) in an initial recognition event between two membranes. This then provides a scaffold on which a number of soluble proteins (Sec1, 2, 3, 4, 5, 6, 8, 9, 10 and 15p) may be involved in addition to the previously identified 'fusogenic proteins' Sec18p (NSF) and Sec17p (α SNAP) to form a protein complex which facilitates the final bilayer fusion event. This process may be further regulated by Sec4p in a mechanism common to intracellular fusion events within membrane trafficking *in vivo* (see **Figure 1.3**). Specific functions have not been identified for proteins involved within this late acting complex and they may represent effector components that regulate the cycling of Sec4p between a membrane bound (active) conformation and a soluble (inactive) conformation and in doing so regulate the actual fusion event itself.

Figure 1.3 Regulation of vesicle docking/fusion by small GTP binding proteins.

Proposed model for the regulation of vesicle fusion events by small GTP binding proteins. This model represents a basic concept of this fusion process in a constitutive secretory system which may be regulated further by a number of GTP activating and inhibitory proteins. Regulated secretion (such as seen at the synaptic cleft) may also be under the control of calcium-binding proteins such as synaptotagmin.



This type of interaction can also be postulated between the ER and Golgi apparatus at which stage the small GTP binding protein Ypt1p acts, possibly in conjunction with the syntaxin-like protein Sed5p (Hardwick and Pelham, 1992), and between the late Golgi compartment and the vacuole where the syntaxin-like protein Pep12p (Becherer and Jones, 1992) has been identified. That a similar mechanism could regulate these events is suggested by the identification of Sec1p-related proteins Sly1p and Slp1p (Aalto *et al.*, 1992) which function in intracellular transport between the ER and Golgi apparatus (Dascher *et al.*, 1991) and from the Golgi to the vacuole (Wada *et al.*, 1990) respectively. The first hint of a related mechanism within mammalian cells has been given with the identification of a Sec1-related protein, Munc18p (Hata *et al.*, 1993).

Once fusion has occurred both v- and t-SNAREs are on the same membrane and must be recycled to their appropriate compartments. SNAREs may also be found in an inappropriate membrane during their initial biosynthesis as they are directed to their final point of action within the cell. It may be that small GTP-binding proteins act as a molecular proof-reading mechanism which only favours correct vesicle interactions through correct SNARE interactions.

Yet basic biochemical evidence still has to be found concerning the details of exactly which v/t-SNAREs recognise and physically interact together, how this interaction is affected by NSF and SNAP and how the small GTP-binding proteins regulate this interaction. What of the stages leading up to membrane fusion that had previously been identified as prefusion complexes that underwent a maturation process before fusion actually occurred (Wattenberg *et al.*, 1986)? Some of these questions are now beginning to be addressed for proteins involved at the synaptic cleft during neurotransmitter release (Sollner *et al.*, 1993b) using *in vitro* reconstitution assays.

1.4 Outline of project

The initial aim of my project was to identify proteins which interacted with the Sec18p from *S. cerevisiae*. The Sec18p had been identified as a homologue of the mammalian protein NSF and so was implicated in membrane fusion events. A genetic interaction had been shown between the *SEC18* gene and the *SEC17* gene (α SNAP homologue) although no other soluble/membrane proteins had been identified. Two approaches were taken: first, affinity purification of interacting proteins using a recombinant Sec18p fusion protein encoded by a chimaeric gene consisting of the complete ORF of the *SEC18* gene and a DNA fragment encoding three IgG binding domains of *Staphylococcus aureus* Protein A moiety. A genetic approach was also used to produce dominant negative mutants of the *SEC18* gene to screen for novel genes that interact with the *SEC18* gene. This latter approach proved to be more successful in that a number of mutants were isolated with novel phenotypes.

Chapter 2
Materials and Methods

Materials

2.1 Chemicals / antibodies / enzymes

Most materials were obtained from BDH Chemicals, Sigma Chemical Co., or Fisons Ltd. [α - 35 S]-dATP (>600 Ci/mmol) was obtained from Amersham International plc. Fast Flow IgG Sepharose was obtained from Pharmacia and Poly-Prep columns from Bio-Rad. The CPY antibody was kindly supplied by Dr. N Bryant and the Sec18p and Sec17p antibodies were raised as described in Appendix to Chapter 3.

2.2 Bacterial / yeast strains and plasmids

Strains of *E. coli* and *S. cerevisiae* used in this study are described in **Table A.1 and A.2** of the **Appendix**. Plasmids used within this study are also presented in the **Appendix** in **Table A.3 and A.4** with a description of their origin.

2.3 Bacterial plates / media

In general all bacterial cultures were grown in Luria Broth (L-Broth) consisting of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl supplemented with the appropriate antibiotic (ampicillin or kanamycin) to a final concentration of 15 μ g where necessary. For blue/white colony selection the medium also contained 15 μ M IPTG and 0.005% X-gal. Addition of 2% (w/v) agar to this medium gave plates.

In order to maintain its F' plasmid, the bacterial strain NM522 was stored on M9 minimal plates which were made as follows : 2% (w/v) agar and 0.4% (w/v) glucose were autoclaved and left to cool slightly. 100 X additive (0.1 M magnesium sulphate, 0.01 M calcium chloride) and 10 X M9 salts (7% (w/v) sodium phosphate (dibasic), 3% (w/v) potassium phosphate (monobasic), 0.5% (w/v) NaCl, 1% (w/v) ammonium chloride) were added to a final concentration of 1 X and this was supplemented with 0.0002% vitamin B₁.

2.4 Yeast plates / media

Yeast cultures were generally grown in YEPD medium (rich medium) containing 1% (w/v) yeast extract, 2% (w/v) bacto peptone, 2% glucose. Selective medium was also used containing 0.7% (w/v) yeast nitrogen base (without amino acids), either 2-3% (w/v) glucose (SD), 2-3% (w/v) galactose (SG) or 2-3% (v/v) glycerol/ethanol as a carbon source and the appropriate amino acids at a final concentration of; 20 µg/ml histidine, 30 µg/ml leucine, 20 µg/ml uracil, 20 µg/ml tryptophan. The addition of 1% (w/v) casamino acids (supplemented with uracil), which are produced commercially by the acid-hydrolysis of casein (a milk protein), was also used as it contains most of the common amino acids and is rich in the essential ones. Again addition of 2% (w/v) agar gave plates.

Yeast sporulation plates were used and basically contained a high carbohydrate and low nitrate medium consisting of 1% (w/v) potassium acetate, 0.1% (w/v) yeast extract, 0.05% (w/v) glucose, 2% (w/v) agar.

Methods

2.5 DNA manipulations

All general DNA techniques such as restriction endonuclease digestion, extraction with phenol/chloroform, precipitation in ethanol and ligations were performed as described by Sambrook *et al.* (1989).

Gel electrophoresis, for the separation and visualization of DNA fragments was carried out using agarose gels between 0.4% and 1% (w/v) agarose; DNA fragment size was estimated by comparing their mobility through an agarose gel with those of fragments of known size liberated by digestion of phage λ DNA with *Bst* EII or present in a commercially available 1 kbp DNA ladder.

2.6 Transformation of bacterial and yeast cells

DNA was transformed into bacterial cells which had been treated with CaCl_2 as described by Sambrook *et al.* (1989). Transformation of LiOAc treated yeast cells, using single stranded carrier DNA was performed as described by Geitz *et al.* (1992).

2.7 Stock preservation

All bacterial and yeast cultures were preserved in 15% (v/v) glycerol and stored at -70°C .

2.8 Random Mutagenesis

(a) Mutagenic polymerase chain reaction (PCR)

Mutagenic PCR was performed essentially as described by Muhlrads *et al.* (1992). The PCR reaction contained the following components: 10ng DNA template ; 1X reaction buffer (10 X stock : 1 M Tris.HCl pH8.0; 5 M KCl; 1% (v/v) Triton X100); 1 mM each dGTP, dCTP and dTTP; 200 μM dATP; 2.5 units of *Taq* DNA polymerase (Promega). The reaction was first optimised for MgCl_2 concentration (from 1.5 mM to 6 mM) measured by the appearance of a PCR product. An appropriate MnCl_2 concentration was then added. Reactions were carried out in a final volume of 100 μl and overlaid with 50 μl mineral oil.

(b) Propagation in the *mutD* bacterial strain.

The *E. coli mutD* bacterial strain has a deficient DNA proof-reading ability (Echols *et al.*, 1983) and is resistant to the antibiotic rifampycin. The plasmid to be mutagenised was transformed into the *mutD* strain. Transformed cells were incubated for a further 40 min at 37°C in L-Broth during a recovery period then transferred into a 50 ml L-Broth culture containing the appropriate antibiotic and shaken at 37°C overnight. Mutagenised DNA was recovered from this culture using standard DNA procedures.

2.9 Rapid isolation of plasmid and chromosomal DNA from yeast (Hoffman and Winston, 1987)

This protocol describes the isolation of DNA from the yeast *S. cerevisiae* which can be used in the transformation of *E. coli* for plasmid rescue. This procedure also releases chromosomal DNA which following a further two ethanol precipitations can be digested by restriction endonucleases and analysed by Southern blot analysis.

The yeast strain was grown in 5 ml of YEPD medium overnight until the stationary phase of growth. 1.5 ml of this culture was harvested in a microfuge tube and the supernatant removed. The cell pellet was resuspended in any residual liquid and 0.2 ml of lysis buffer (2% Triton X100, 1% SDS, 100 mM NaCl, 10 mM Tris.HCl pH8.0 and 1 mM EDTA) added. A further 0.2 ml phenol / chloroform : isoamyl alcohol (24 : 1) and 0.3g of glass beads were added to the resuspended yeast pellet. This suspension was vortexed for 2 min (this step is important as further vortexing may lead to shearing of chromosomal DNA) then centrifuged for 5 min in a bench top microfuge at 15,000 rpm. The upper aqueous layer was removed and DNA was ethanol precipitated from this and resuspended in 50 µl of TE buffer. 10 µl of this DNA was used to transform 200 µl of competent *E. coli* cells. For Southern blot analysis at least 15 µl of this DNA was digested.

2.10 Southern hybridisation

Transfer of DNA from agarose gels to a nylon Hybond H⁺ filter and subsequent hybridization of a nonradioactively digoxigenin labelled probe to the filter were carried out essentially as described by Sambrook *et al.* (1989) using a method based on that of Southern (1975). Approximately 3 µg of purified DNA was labelled following the method described by Boehringer Mannheim Biochemica: DNA synthesis was primed on denatured DNA by random hexadeoxynucleotides in the presence of the Klenow fragment of *E. coli* DNA polymerase I and the nucleotide analogue digoxigenin-11-

dUTP. Detection of the digoxigenin-labelled probe was with an anti-digoxigenin-alkaline phosphatase conjugate which was visualized by an enzyme linked colour reaction.

2.11 DNA sequence analysis

Two methods of sequencing were employed in this work, one based on single-stranded M13 (as used in Chapter 3) and the other based on double stranded plasmid DNA (as used in Chapter 4). Single-stranded M13 DNA was prepared as described in Sambrook *et al.* (1989) and the sequencing reactions were performed as described in the BRL 'M13 cloning/dideoxy sequencing instruction manual' using a BRL sequencing kit. Double stranded DNA sequencing reactions were performed as described in the 'USB Sequenase version 2.0' manual. Both methods employed the dideoxy chain termination method of Sanger *et al.* (1977) for sequencing in the presence of [$\alpha^{35}\text{S}$]-dATP. The sequencing reactions were electrophoresed on 6 - 8% acrylamide wedged gels containing 8 M urea.

2.12 Expression of Protein A fusion proteins from the P_{lac} promoter.

The *E.coli* strain NM522 transformed with either a pKpra or a pAX11, 12 or 13 derived plasmid was grown in 100ml of selective medium (L-Broth containing the antibiotic, kanamycin) at 37°C to an O.D₆₀₀ of 0.6. To induce expression from the *lac Z* promoter IPTG was added to a final concentration of 0.25 mM and the cells were grown for a further 2 - 4 hrs.

2.13 Expression of β -galactosidase fusion proteins from the P_R promoter.

The *E.coli* strain pop2136 transformed with a pEX11, 12 or 13 derived plasmid was grown in 100 ml of selective medium (L-Broth containing the antibiotic, ampicillin) at 30°C to an O.D₆₀₀ of 0.5. To induce expression from the P_R promoter of

bacteriophage λ the cells were then incubated at 42°C for a further 4 - 5 hrs.

2.14 Fractionation of bacterial cultures by centrifugation.

The fractionation of induced bacterial strains was similar in all cases. Cells were harvested by centrifugation at 8,000 rpm for 8 min then the cell pellet was washed once with 0.1 M Tris.HCl pH7.4 and resuspended in 5 - 10 ml of this buffer. Lysozyme was added to a final concentration of 1 mg/ml and this suspension was placed on ice for 20 min. The yeast cells were lysed by sonication (4 X 30sec bursts with 2 min intervals on ice between each burst). The suspension was then fractionated; an initial low speed clearing spin of 3,000 rpm for 5 min yielded a pellet (P3). The resultant supernatant was subjected to a further centrifugation at 20,000 rpm for 20 min to give an insoluble (P20) and a soluble (S20) fraction. Both the P3 and P20 fractions were resuspended in a volume similar to the S20 fraction (5 - 10 ml) and analysed by SDS PAGE and subsequent Western blot analysis. Using this method β -galactosidase fusion proteins tended to fractionate in the P3 fraction whereas protein A fusion proteins fractionated in the S20 fraction.

2.15 Use of Fast Flow IgG Sepharose for the purification of Protein A fusion proteins.

The basic method used for purification of protein A fusion proteins from either bacterial or yeast cultures is as follows: 1 ml of IgG Sepharose (wet volume) was equilibrated, by washing (4 times) with TST buffer (50 mM Tris.HCl pH7.4, 150 mM NaCl, 0.05% Tween-20). The bacterial or yeast extract was then added to this equilibrated IgG Sepharose and incubated overnight on a rotating wheel at 4°C. This suspension was then placed in a 'Poly-Prep' chromatography column and the unbound material was allowed to flow through. The column was washed with TST buffer until the OD₂₈₀ of the wash coming through the column was zero. 5 - 10 ml of elution buffer (0.5 M acetic acid adjusted to pH3.4 with ammonium acetate) was then

added and the eluted material collected and dialysed against 5 L of distilled water overnight at 4°C. Samples were then frozen at -70°C and freeze dried and resuspended in an appropriate volume of buffer.

2.16 Yeast glass bead homogenate

The yeast strain was grown in selective medium until the desired OD₆₀₀ was attained at which point the cells were harvested (5,000 rpm for 5 min). This cell pellet was washed once with distilled water then resuspended in the same volume of Lysis buffer (0.1 M Tris.HCl pH7.0, 2 mM EDTA pH7.4, 20 mM PMSF, 20 mM EGTA, 10 µg/ml each of pepstatin, leupeptin, chymostatin, and antipain). An equal volume of glass beads was added and this suspension was vortexed for 4 X 2min with 2min intervals on ice. This suspension was spun out through the glass beads at 3,000 rpm for 10 - 15 min to remove broken yeast cell debris and give a crude yeast lysate.

2.17 Fractionation of crude yeast cell homogenate

Where appropriate the crude yeast cell lysate was further fractionated by centrifugation at 12,000 rpm for 15 min to give a low speed pellet (P12). The supernatant was further centrifuged at 50,000 rpm (100,000 x g) for 1 hour in a Beckman TL100 rotor to yield high speed pellet (P100) and supernatant (S100) fractions.

2.18 Chemical mutagenesis of yeast cells using EMS

The yeast strain was grown in 10 ml of YEPD medium overnight at 30°C and 2.5 ml of the culture was harvested. The yeast cell pellet was washed twice in 50 mM potassium phosphate buffer pH7.0 and resuspended in a final volume of 10 ml. 300 µl of EMS was added to this suspension in a sterile screw top glass bottle and vortexed as EMS is poorly miscible in water. This suspension was incubated at 30°C for 30 min at which point the action of EMS was arrested by adding an equal volume

of 10% (w/v) of filter-sterilised sodium thiosulphate. The cells were then harvested and washed twice with sterile distilled water. The yeast cells were then incubated in YEPD medium for several generations of growth in a recovery period before plating out onto selective agar plates and growth at 25°C. This treatment generally resulted in a 50 -90% lethality.

2.19 Construction of yeast strains and random spore analysis

Yeast strains were constructed within this work by mixing two haploid strains and growing them overnight at 25°C first on YEPD plates. The strains were then replica-plated onto selective plates and grown at a temperature which only allowed the growth of diploid colonies. Yeast strains that grew under such conditions were then sporulated, by growth on sporulation plates (see Section 2.4) at 25°C for up to 7 days. The extent of sporulation was monitored by visualization under a light microscope. Once sporulation had occurred the ascus wall was removed to allow the propagation of individual spores.

As many cells as possible were scraped off from a sporulation plate and resuspended in 200 µl of sterile distilled water. 40 µl of a 5 mg/ml zymolyase solution was added and this suspension incubated at 30°C for 1 hr. 0.1g of glass beads was added along with 500 µl of sterile distilled water and this was vortexed for 30 seconds. Dilutions of this spore suspension were then plated out onto YEPD agar plates and incubated at 25°C to allow germination, yielding individual haploid yeast colonies.

2.20 Determination of yeast mating type

Yeast strains of unknown mating type were individually mixed with the two strains DC14 *MAT α ; hisI* and DC17 *MAT α ; hisI* under conditions that selected for growth of prototrophic diploids. Only yeast strains of opposing mating types will form such diploids.

2.21 Processing of yeast cells for viewing under the Electron Microscope using LR white resin.

(a) Fixation of the yeast cells

To avoid any morphological changes of the yeast cells when subjected to centrifugation the cells were fixed directly in the growing yeast culture. A 1/10 volume of 10X prefixative solution (10% glutaraldehyde, 2% methanol free formaldehyde, 0.4 M potassium phosphate pH7.0) was added to the growing yeast culture and allowed to sit at room temperature for 5 min. The cells were then pelleted by centrifugation at 5,000 rpm for 5 min and resuspended in 1/10 volume of ice cold 1X prefixative solution and allowed to complete fixation for 30 min at 4°C. Excess fixative was removed by three phosphate buffer washes (0.04 M potassium phosphate pH7.0) leaving the cell suspension in each change of buffer for at least 5 min.

(b) Periodate treatment of fixed yeast cells

The washed cell pellet was resuspended in 1ml of freshly prepared 1% sodium metaperiodate (aqueous) and incubated at room temperature for 15 min. The samples became clumpy at this point and were pelleted by centrifugation at 5,000 rpm for 5 min and washed once in phosphate buffer. The cell pellets were resuspended in 50 mM ammonium phosphate to block free aldehyde sites and were incubated at room temperature for 15 min before being washed twice with distilled water.

(c) Dehydration of yeast cells

Periodate treated cells were resuspended in 1 ml of 50% ethanol and then pelleted by centrifugation at 5,000 rpm for 5 min after which they were resuspended in 1 ml of 70% ethanol. At this point cells formed firm clumps which could be dispensed into microfuge tubes containing 70% ethanol and stored as aliquots at -20°C for several months. However, in this work processing was carried out immediately. The tubes containing the samples were placed upright on a rotating wheel with their lids open for

5 min at room temperature. Dehydration was completed by one 5 min incubation in 95% ethanol followed by a further three incubations in 100% ethanol (a previously unopened bottle).

(d) Infiltration of dehydrated yeast cells with LR white resin

The last 100% ethanol incubation was replaced with a solution of 2 parts ethanol to 1 part LR white resin and the samples were returned to the rotating wheel for 1 hour (in a fume hood). The solution was replaced with a 1 : 1 ethanol : resin mix and the samples rotated for another hour. The samples were incubated, uncapped, in a fresh 1 : 1 resin mix overnight on the rotating wheel in the fume hood (this allows the ethanol to slowly evaporate thus increasing the resin concentration gradually). The following day the residual resin was replaced with fresh 100% LR white resin and the samples were rotated for 1 hour. The resin was then replaced and the samples placed under vacuum (20 psi) for 15 min to be degassed before being placed back on the rotating wheel for a further hour.

(e) Embedding of yeast cells in solid LR resin

Small clumps of cells were removed from the samples using a tooth pick and placed into gelatin capsules filled with LR white resin. The sample was allowed to sink to the bottom of the capsule and ideally was positioned in the centre. The capsules were then placed under vacuum for 15 min and polymerization of the resin was achieved by leaving sealed capsules in an incubator at 45°C-50°C for approximately 2 days.

(f) Grid preparation, sectioning and staining

Sections from the LR white resin blocks were cut by the Department of Pathology, University of Edinburgh and placed onto gold EM grids. The sections were air dried on the grids for 2 days before staining. The grids were stained in 2% aqueous uranyl acetate for 1-5 min then in Reynolds lead citrate for 30 seconds.

2.22 Electrophoretic separation and detection of proteins

Separation of proteins was achieved by electrophoresis through polyacrylamide gels following the basic procedure of Laemmli (1970). Polyacrylamide gels ranging from a 7.5 - 12% (w/v) separating gel with a 5% (w/v) stacking gel were normally analysed using the Hoeffer 'tall mighty small' gel apparatus using the following solutions:

I. Separating gel buffer

0.75 M Tris.HCl pH8.8, 0.2% (w/v) SDS

II. Stacking gel buffer

0.25 M Tris.HCl pH6.8, 0.2% (w/v) SDS

III. Acrylamide solution

40% (w/v) acrylamide, 0.67% (w/v) *N,N'*-methylenebisacrylamide

or 44% (w/v) acrylamide, 0.3% (w/v) *N,N'*-methylenebisacrylamide (**Figure 5.5**)

IV. Electrophoresis buffer

0.125 M Tris., 0.2 M glycine, 0.1% (w/v) SDS (final pH of solution is 8.3 with no adjustment)

V. SDS sample buffer

0.0625 M Tris.HCl pH6.8, 20% glycerol, 4% (w/v) SDS, 5% (w/v) β -mercaptoethanol

Proteins were visualized after electrophoresis by either Coomassie Blue staining or Silver staining:

(a) Coomassie Blue staining

Gels were soaked in a solution of 0.25% (w/v) Coomassie Brilliant Blue dissolved in 50% (v/v) methanol, 7% (v/v) acetic acid for approximately 15 min. Gels were destained in a solution of 10% (v/v) methanol, 7% (v/v) acetic acid.

(b) Silver staining

Gels were soaked in 50% (v/v) methanol for at least 1 hr. The silver stain was made fresh as follows: Solution (a) 0.8 g of silver nitrate was dissolved in 4 ml of distilled water. Solution (b) 21 ml of 0.36% sodium hydroxide was added to 1.4 ml of 14.8 M ammonia. Solution (a) was added dropwise to solution (b) with constant stirring and this was made up to a final volume of 100 ml with distilled water. The gel was soaked in this solution for 15 min with gentle agitation. The gel was subsequently washed for at least 5 min in distilled water during which time 100 ml of developer was freshly made (0.005% (v/v) citric acid, 0.019% (v/v) formaldehyde). The gel was developed by soaking it in developer until bands appeared on the gel (usually within 10 min of adding the developer). The developing was stopped by washing the gel in distilled water then placing / storing it in destain solution.

2.23 Semi-dry blotting of proteins

Proteins were transferred to a nitrocellulose filter (Hybond C extra) using a semi-dry blotting technique employing the following buffers :

Anode buffer 1: 0.3 M Tris., 20% (v/v) methanol, 0.1% SDS: pH10.4

Anode buffer 2: 25 mM Tris., 20% (v/v) methanol, 0.1% SDS: pH10.4

Cathode buffer: 25 mM Tris., 20% (v/v) methanol, 0.1% SDS: 40 mM
6-amino-*N*-hexanoic acid: pH9.4

Six pieces of Whatman 3mm paper and one piece of nitrocellulose membrane were cut to the same size as the gel to be blotted. Two pieces of Whatman 3mm paper were soaked in anode buffer 1, one piece in anode buffer 2 and three pieces along with the gel in cathode buffer. The nitrocellulose membrane was soaked in distilled water. These were layered as follows onto the semi-dry blotting apparatus: 2 X pieces anode 1, 1 X pieces anode 2, nitrocellulose membrane, polyacrylamide gel then 3 X pieces cathode. The area of the gel was measured and it was blotted for 1 hour at 0.8mA X [area of gel] cm².

2.24 Western blot detection of proteins.

After transfer of proteins onto a nitrocellulose membrane nonspecific sites on the membrane were blocked by incubating the membrane for 1 hr in blocking buffer (5% non-fat dried 'Marvel' powdered milk, in TBST buffer: 1% Tween-20, 10 mM Tris.HCl pH7.4, 150 mM NaCl). Primary antibody was then added into this solution at an appropriate dilution and incubated for a minimum of 1hr to an overnight incubation with gentle agitation. Excess primary antibody was removed by washing the membrane 2 X 15 min then 2 X 5min washes in TBST buffer. The membrane was then incubated in blocking buffer and incubated with the second antibody (usually a donkey anti-rabbit HRP conjugate) at a dilution of 1 : 5,000 for 20 min. The membrane was washed as described above before detection by the Enhanced chemiluminescence (ECL) detection system (Amersham).

2.25 Isolation of antigen and the immunization of rabbits for antibody production

Female New Zealand white rabbits were immunized with bacterially expressed and purified protein A fusion proteins. These fusion proteins were expressed and purified from the bacterial strain NM522 (as described in Sections 2.12 and 2.15). The IgG Sepharose purified material could either be directly used to immunise a rabbit if the material was free of contaminating proteins or the protein of interest could be purified further by electroelution from an SDS polyacrylamide gel as follows: The sample was loaded onto an appropriate percentage SDS polyacrylamide preparative gel lane which has a maximum capacity of 1 ml of sample. After the gel was run (25 mA for 5 hrs or 50 V overnight) it was stained in Coomassie Blue stain for a short period. The appropriate band was excised, after destaining the gel, as a strip of polyacrylamide which was cut into a number of smaller pieces. These fragments were placed in dialysis tubing containing 5 ml of electrophoresis buffer and placed in a tank of this buffer and subjected to 125 V for 5 hrs. The buffer containing the electroeluted

protein was then removed and dialysed against distilled water overnight at 4°C.

At least 100 µg of purified protein A fusion protein (in a volume of approximately 150 µl) was mixed to form an emulsion with an equal volume of Freund's complete adjuvant (1 mg *Mycobacterium tuberculosis*, heat killed and dried, 0.85 ml paraffin oil and 0.15 ml mannide monooleate per ml of adjuvant) and this was then injected subcutaneously into the rabbit. A second injection was performed six weeks later but in this and subsequent injections Freund's incomplete adjuvant (0.85 ml paraffin oil and 0.15 ml mannide monooleate per ml of adjuvant) was used in the emulsion. A 10 - 40 ml bleed was obtained from the rabbit six days after the third injection. The blood was left overnight at 4°C to clot and the serum was collected by centrifugation at 3,000 rpm for 15 min. The supernatant was filtered through a 0.22 µm Acrodisc and sodium azide added to a final concentration of 0.1%. The serum was stored at -20°C until needed further. If the antibody titre was high in a rabbit after its 5 - 6th boost injection then that animal was sacrificed and the serum was stored at -20°C.

2.26 Affinity purification of polyclonal antibodies

2 ml of Biorad Affi-gel 10 (wet volume) was washed once with 10 ml distilled water then twice with 10 ml 0.1 M Hepes pH7.4 (each time allowing the gel to settle out on ice between washes). Typically 500 µg of β-galactosidase fusion protein resuspended in 5 ml of 0.1 M Hepes buffer pH7.4 was bound overnight at 4°C on a rotating wheel. The suspension was transferred to a 'Poly -Prep' Chromatography column and washed with 0.1 M Hepes buffer pH7.4 until the O.D₂₈₀ of the wash was zero. Any uncrosslinked sites on the gel were blocked by incubation of the column in 1 M ethanolamine on a rotating wheel at 4°C for 1 hr. The ethanolamine was washed from the column again with 10 ml 0.1 M Hepes buffer pH7.4.

Immune serum (10 - 20 ml) was then added to this affinity column and it was



incubated overnight on a rotating wheel at 4°C. Unbound material was collected and then the column was washed using 0.1 M Hepes pH7.4 until the O.D₂₈₀ of the wash was zero. The bound antibodies were then eluted from the column using 5 ml of 0.2 M glycine pH2.8. 1 ml fractions of this eluted material were collected in microfuge tubes containing 75 - 100 µl of 1 M Tris. HCl pH8.0 to neutralise the final solutions. The O.D₂₈₀ of each fraction was monitored and the peak of protein concentration (containing the affinity - purified antibodies) was aliquoted out into 100 µl fractions containing a final concentration of 0.1% sodium azide and stored at -20°C.

2.27 Detection of β -lactamase activity

β -lactamase activity was measured spectrophotometrically by following a colour change of the substrate at wavelength of 490 nm using the reagent nitrocefin. A stock solution of nitrocefin was prepared containing 5 mg nitrocefin dissolved in 0.5 ml of DMSO and this was made up to a final volume of 10 ml by the addition of 9.5 ml of 0.1 M sodium phosphate buffer (pH7.0) and was stored at 4°C. β -lactamase activity was measured by adding 50 µl of sample to 0.95 ml of 0.1 M sodium phosphate solution pH7.0 and 50 µl of nitrocefin stock solution and following the change in absorbance read at 490 nm.

Chapter 3

***Production of a Sec18-Protein A protein
for use in attempts to affinity purify Sec18-
containing complexes***

3.1 Introduction

Over the years a number of proteins have been identified that may be involved in specific targeting and fusion events occurring during intracellular membrane traffic. The *N*-ethylmaleimide-sensitive fusion protein (NSF) was first implicated in membrane fusion by its ability to restore intercisternal Golgi transport in a mammalian cell-free assay system (Block *et al.*, 1988, Malhotra *et al.*, 1988). NSF requires additional soluble NSF attachment proteins (SNAPs) and membrane bound receptors to attach to Golgi membranes forming a multisubunit complex with a sedimentation coefficient of 20S. Recent data identified components of this 20S particle from bovine brain, involved in synaptic vesicle release, using a functionally active recombinant form of NSF which was C-terminally tagged with the c-myc peptide and purified from *E. coli* (Sollner *et al.*, 1993).

A biochemical approach to the isolation of proteins that interact with the yeast homologue of NSF encoded by the *SEC18* gene was attempted. In the absence of a suitable antibody against Sec18p for good immunoprecipitation analysis a Protein A tag was attached to the carboxy-terminus of Sec18p. Protein A is a 42 kDa polypeptide that is a normal constituent of the cell wall of *S. aureus* and is known to bind to the F_C domain of antibody molecules. It is essential for this type of approach that the tagged protein is still biologically active and the tag is not so large as to cause steric hindrance to complex formation and normal functioning of the protein *in vivo*. This chapter presents the steps taken in constructing a hybrid gene encoding a Sec18pra fusion protein in a yeast expression system. The functional activity of the hybrid protein was tested relative to the wild-type activity and I attempted to affinity purify proteins that complex to Sec18p *in vivo* using various conditions of isolation.

3.2 Construction of a C-terminal tagged version of Sec18 protein

A series of vectors (Geitz *et al.*, 1988) allowing fixed copy levels within yeast are

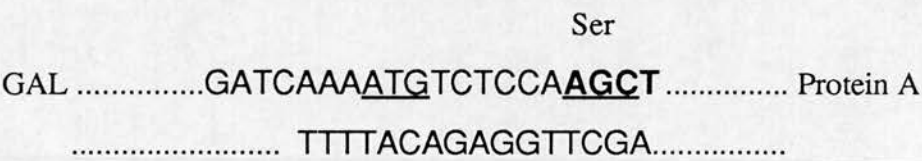
commonly used in our laboratory. YEp (yeast episomal plasmid) based vectors contain the 2 μ m circle allowing them to have stable, high copy propagation. YCp (yeast centromeric plasmid) based vectors carry a chromosomal centromere allowing the plasmid to engage the spindle apparatus of the cell and so give 1-2 copies per cell, and much greater stability than YEp plasmids. The plasmids YEpGAL and YCpGAL (see **Table A.3 and A.4**) both containing the *TRP1* gene as a selectable yeast marker, were used throughout to give two levels of expression of proteins within yeast when grown on galactose inducing medium, the *GALI* promoter being repressed in the presence of glucose in the medium (St. John and Davis, 1981; Schultz *et al.*, 1987).

To create a gene fusion between the *SEC18* gene and Protein A - encoding DNA a new restriction site was required at the 3' end of the *SEC18* gene to facilitate the cloning strategy and produce an in-frame fusion protein. The complete open reading frame (ORF) of the *SEC18* gene including 70 nucleotides of upstream sequence was cloned into a phage vector to allow the introduction of an *FspI* site by the Kunkel method for site directed mutagenesis as described in **Figure 3.1**. This fragment should contain enough information for the expression of the *SEC18* gene as sequences upstream from the transcription initiation site do not seem to be implicated in gene expression and the promoter elements are thought to function from within the *SEC18* gene itself (Eakle *et al.*, 1988).

The plasmid pKpra was used as a source of Protein A-encoding DNA. This plasmid encodes a Pra - *lac Z* fusion protein of 26 kDa that contains an N-terminal portion of Protein A consisting of two and a half IgG binding domains of approximately 16.9 kDa. This portion of Protein A was used both to produce a C-terminal Sec18pra in-frame fusion and to construct a Pra control to use in future affinity purification analysis. Firstly, two stop codons (TAA,TAA) and a new *XhoI* restriction site were

introduced at the 3' end of the Protein A fragment to ensure the correct translational termination of the Sec18pra peptide and to facilitate the cloning strategy respectively producing the construct pKpra2 (**Table A.4**). This modified Pra fragment was subcloned into the phage construct M13SEC18(*Fsp*I) at the 3' end of the *SEC18* gene producing an in frame fusion product which was subsequently subcloned into the yeast vectors YCplac22, YEplac112, YCpGAL and YEpGAL (see **Table A.3**). The resulting plasmids pCH1 and pCH2 contain a *SEC18pra* fusion gene under the control of the *SEC18* promoter whereas pCH3 and pCH4 contain the same gene under the control of the strong *GAL1* promoter. The *SEC18* gene has been shown to produce two gene products of approximately 82 kDa and 84 kDa (Eakle *et al.*, 1988), therefore it was expected that each construct would encode two gene products of predicted sizes of approximately 99 kDa and 101 kDa.

A *Hind*III/*Xho*I cassette containing a portion of the Pra gene (used for the Sec18pra fusion construct) was cloned into YCpGAL and YEpGAL. Two oligonucleotides were used which facilitated the cloning strategy and also allowed the in frame fusion of the Pra fragment into the plasmids such that it was under the control of the *GAL1* promoter (as shown below) and produced the two constructs pCH5 and pCH6 respectively which encode a product of 16.9 kDa (see **Table A.4**).



This allowed the study of the effects that expressing just the Pra tag within yeast cells had in the subsequent affinity purification analysis so distinguishing between non-specific and specific binding of proteins to the Sec18p portion of the hybrid.

3.3 Detection of active fusion protein *in vivo*

An essential prerequisite for any tagged version of a protein is that the 'tag' does not interfere with the activity of the protein *in vivo*. The yeast strain CHY01 (see **Table A.2**) has a temperature sensitive mutant *sec18-1* allele and cannot grow at the nonpermissive temperature of 37°C. The Sec18pra fusion protein should be able to complement this defect when expressed in the yeast strain CHY01 at 37°C if it functions *in vivo*.

CHY01 was transformed with plasmids pCH1, 2, 3, and 4 to give the strains CHY01(pCH1 to 4). Growth of these strains was monitored on both glucose (SD) and galactose (SG) selective media at the permissive (25°C) and restrictive (37°C) temperatures and compared with that of an untransformed strain (**Table 3.1**). Yeast strains CHY01(pCH1) and CHY01(pCH2) show growth at the restrictive temperature on glucose selective medium and slight growth on galactose. This suggests that the internal promoter element within the *SEC18* gene can produce enough fusion transcript to encode Sec18pra fusion protein which is active *in vivo* thus complementing the *sec18-1* mutation. Yeast strains CHY01(pCH3) and CHY01(pCH4) complement the *sec18-1* mutation on galactose medium at 37°C and slightly on glucose medium at 37°C suggesting again that there is slight leakiness in the control of the expression of the Sec18pra fusion protein due to the effect of the internal promoter in the *SEC18* gene itself. Complementation of the *sec18-1* mutation by both high and low copy number yeast vectors suggests that it is not an abundant degradation product lacking the protein A moiety that is acting *in vivo* but the full length fusion protein.

3.4 Fractionation studies showing the intracellular distribution of fusion protein

Since the fusion protein complements the *sec18-1* mutation it would suggest that it

was active *in vivo* and so should have a similar intracellular distribution to that of the wild-type protein. Yeast cells expressing the fusion construct were lysed using glass beads and fractionated in the presence or absence of ATP by differential centrifugation closely following the fractionation procedure used in the characterization of Sec18p (Eakle *et al.*, 1988). Each fraction was analysed under denaturing conditions using SDS PAGE and the distribution of the hybrid protein between the various fractions was monitored by Western blot analysis (**Figure 3.2(a) and (b)**). The Sec18pra protein was found to fractionate in a similar manner in the presence or absence of ATP within the medium. Significant amounts of protein were detected in the P12 fraction which has been shown to contain significant amounts of ER / Golgi complex. Also the hybrid protein fractionates evenly between the high speed membrane pellet (P100) containing transient transport vesicles and the supernatant (S100) fraction. Unmodified Sec18p has been reported to fractionate mainly between the P100 and S100 fractions with little association seen with the P12 fraction (Eakle *et al.*, 1988). Therefore the presence of the fusion protein in the P12 fraction seen in this study could either be an artifact caused by the overexpression of this fusion protein which may associate with other intracellular membranes or the Pra tag may be causing the aggregation of the fusion protein. However, the fact that there is fusion protein in the P100 / S100 fractions supports the conclusion that a proportion of the hybrid protein is showing a 'wild-type' intracellular distribution.

Two forms of the fusion protein were resolved using 7.5% SDS PAGE and were found in significant amounts in both the 100,000xg pellet (P100) and supernatant (S100) fractions as reported for the wild-type protein (**Figure 3.2 (c)**). Treatment of the yeast extracts with 0.1M sodium bicarbonate pH12.5 prior to fractionation removed the fusion protein from the P100 into the S100 fraction (**Figure 3.3**). This suggests that the fusion protein is a peripherally membrane associated protein. This movement is seen also when extracts are treated with 2.5M urea, suggesting that the

fusion protein is associated with the membrane possibly as a peripheral protein complex / aggregation. The fractionation studies along with the previous complementation analysis suggests that a proportion of the hybrid protein when expressed in yeast is functionally active *in vivo* and can therefore be used to affinity purify proteins that act in a 'fusogenic complex' with Sec18p *in vivo*.

3.5 Initial affinity purification studies of the fusion protein using IgG Sepharose

The Sec18pra fusion protein seems to be active *in vivo* and so should interact with the wild-type Sec18p in functional tetramers that can participate in membrane fusion events within the 20S fusogenic complex. Purification of this complex was attempted using the affinity of the Pra tag for the F_C portion of IgG on a Fast Flow IgG Sepharose column in a single step purification. The basic procedure used to affinity purify proteins that complex with Sec18p is as described in Materials and Methods. A number of variations on this basic experiment were tried in the attempt to identify specific proteins that complexed with Sec18p *in vivo*. All binding studies were carried out using lysates prepared from the transformed yeast strain JRY188(pCH3) and the control strain JRY188(pCH5). Initially the Sec18pra fusion protein was bound from a P100 fraction produced from these strains since the 20S complex is membrane associated and a possible membrane receptor could therefore be identified in this way. Various purifications were done from detergent - solubilised P100 samples (prepared with either 0.1% Triton X100 or RIPA buffer: 150 mM NaCl, 1% Nonidet 40, 0.5% DOC, 0.1% SDS and 50 mM Tris.HClpH8.0). However detection of proteins bound to the hybrid protein was very difficult even on silver stained SDS polyacrylamide gels. Possibly any complexes formed *in vivo* may be transient and dissociate during the affinity purification procedure so making protein detection difficult.

(a) a thiol-reversible crosslinker 3,3'-Dithio-bis (propionic acid Nhydroxysuccinimide ester) (DSP) which has previously been used to isolate membrane bound multi-subunit complexes (Deshaies *et al.*, 1991). To optimise the degree of crosslinking within Sec18pra containing yeast extracts, S100 and P100 fractions were incubated with 0-12 mM DSP for 30 min at on ice. 0.1 M ammonium acetate was added and the extracts incubated for a further 10 min on ice to terminate the crosslinking reaction. The samples were then analysed by SDS PAGE for the disappearance of low molecular weight proteins and the appearance of large complexed bands on coomassie staining of the gels. The appropriate amount of crosslinker (0.5-1 mM) was thus added to either S100 fractions or detergent-solubilized P100 fractions to try and stabilise any complexes involving Sec18pra prior to binding to IgG Sepharose; (b) an ATP regenerating system was also added to the initial yeast extract prepared as the hydrolysis of ATP is thought to destabilise the 20S complex (Wilson *et al.*, 1992) and (c) the time taken to bind the material to the IgG Sepharose was reduced to approximately 1hr at 4°C to minimise complex disassembly. Unfortunately no specifically - bound proteins were identified by these procedures possibly due to the extremely poor recovery of protein from the P100 extracts even using initial large culture volumes (1L). It may be that the P100 material is lacking a stabilising 'cytosolic factor' for complex assembly and so it was decided to try to purify complexes from an S12 yeast extract (which contains both the P100 and S100 fractions). Again, various experiments were designed to purify complexes from the S12 extract of yeast strains expressing either Sec18pra or the control protein Pra. Two main procedures that were employed are presented below:

Procedure I: An S12 extract was prepared from yeast strains JRY188(pCH3) and JRY188(pCH5) and an IgG binding performed in the presence or absence of an ATP regenerating system (**Figure 3.4**). ATP is required for the stability of the soluble form of NSF as shown in a mammalian cell free transport systems. S12 extracts were

incubated at 4°C with IgG Sepharose on a rotating wheel in an attempt to bind complexes that have formed with the Sec18pra fusion protein. Non-specifically bound proteins were removed in an initial wash whereas proteins bound to Sec18pra or the Pra control were eluted from the IgG Sepharose column using a low pH elution buffer (which co-elutes the fusion protein and tag protein) and analysed by SDS PAGE (**Figure 3.4(a)**). The fusion protein and Pra control along with any degradation products were identified by Western blot analysis (**Figure 3.4(b)**). Comparison of Lanes 1 and 3 in **Figure 3.4(a)** shows that there are many proteins that are eluted by a low pH along with the Sec18pra protein and these proteins are also eluted with the Pra control protein and so represent non-specific binding. Comparison of Lanes 2 and 4 show that this non-specific binding is not affected by the presence of an ATP regenerating system. **Figure 3.4(b)** represents a Western blot analysis of these samples. Lanes 1 and 2 show that there is only minor degradation of the Sec18pra fusion protein during the binding procedure and more fusion protein is recovered in the presence of an ATP regenerating system. Although preliminary analysis has shown in this work that ATP does not affect the intracellular distribution of the fusion protein (see **Section 3.4**) the effects of an ATP regeneration system may stabilise the fusion protein in a conformation which is more amenable to binding and therefore show an altered intracellular distribution. Lanes 3 and 4 show that the Pra is fully denatured on SDS PAGE and only one low molecular species is observed. This seems to suggest again that the large amounts of proteins isolated along with the Sec18pra fusion and the Pra control are not degradation products of the fusion protein (lanes 1 and 2 (**b**)) or aggregated Pra complexes (lanes 3 and 4 (**b**)) but proteins which are binding non-specifically to the Protein A tag. One cannot rule out that because there are a number of proteins that are non-specifically binding to the Pra tag, the identification of any specifically-bound proteins that are complexed to the Sec18p are being masked and cannot readily be identified. On repeating this purification a number of times novel protein bands would sometime appear that

seemed to be specific to the Sec18pra fusion protein. However these results were inconsistent and unreproducible. The absence of ATP within the wash buffer may have contributed to the instability of proteins complexed to Sec18pra upon the column. This could be investigated by having a large excess of ATP or an ATP regenerating system present within the TST buffer during the wash procedure.

Procedure II : An affinity purification technique has recently been reported for the isolation of membrane receptors that are responsible for the recognition and docking of the 20S fusogenic complex onto membranes from bovine brain cytosol (Sollner *et al.*, 1993a). This procedure used the observation that in cell free transport systems the 20S complex was membrane associated in the presence of either ATP γ S or of ATP in the absence of any magnesium, and the complex dissociated on the addition of magnesium. A similar approach was attempted using the Sec18pra fusion construct and the control Pra protein as shown in **Figure 3.5(a) and (b)**.

Comparing lanes 1 and 2 (**Figure 3.5(a)**) it can be seen that the addition of magnesium ions led to the elution of a large number of proteins but that these proteins are not specific to Sec18p but rather seem to bind non-specifically to the Pra control also. The fusion protein and Pra control are not eluted in this magnesium wash step as shown in lanes 5 and 6 of **Figure 3.5(b)**. This non-specific binding of proteins to the Pra tag is also observed in lanes 3 and 4 of the low pH washed material in (**a**) this also contains the fusion protein and control Pra as detected in (**b**) in lanes 7 and 8.

There is still the possibility that small amounts of specifically bound proteins are being eluted in the magnesium wash conditions but cannot be detected against a high background of non-specifically bound proteins. To address this question the material eluted by the addition of magnesium was analysed for the presence of wild-type Sec18p and Sec17p using affinity-purified polyclonal antibodies. As shown in

Figure 3.6 Sec18p was shown to be specifically attached to the fusion protein and was not detected in the corresponding Pra control sample. Previous analysis has shown that this material does not contain any fusion construct or related degradation products (**Figure 3.5(a)**) therefore this signal may originate from two other sources; (a) wild-type Sec18p complexed to the Sec18pra fusion protein as part of an active fusogenic complex or (b) association of wild-type Sec18p with the Sec18pra fusion protein in inactive protein aggregates. However, if the fusion protein was binding to the wild-type protein in aggregates it would soon 'mop up' the wild-type protein and the cell would eventually die which is not the case. To confirm the presence of an active complex within the bound material an affinity purified polyclonal antibody was used to identify the presence of Sec17p which has been shown to be involved within the 20S fusogenic complex. However no detectable amounts of Sec17p were present in the magnesium wash material. The lack of Sec17p may be due to the small amounts of this protein present within the bound material compounded by the low titre of the Sec17p antibody compared to that of the Sec18p antibody (see *Appendix to Chapter 3*).

3.6 Conclusion

In this chapter I have described the construction of two gene fusions, one encoding a fusion protein consisting of the yeast Sec18p carrying a C-terminal tag of two and a half IgG binding domains of Protein A from *S. aureus* and the other encoding a Pra control protein, in yeast vectors containing the strong *GAL1* promoter. Although the intracellular distribution of the fusion protein was slightly different from that reported for Sec18p a proportion of Sec18pra did associate with both the high speed soluble (S100) and membrane fractions (P100) as described for the wild-type protein. The *in vivo* activity of the Sec18pra fusion protein was strongly suggested by the finding that it complemented a *sec18-1* mutation at the restrictive temperature. Initial attempts to purify complexes containing the Sec18pra fusion protein revealed that the Pra tag itself

bound a number of proteins nonspecifically and so made the identification of Sec18 specific proteins impossible.

Identification of wild-type Sec18p within the affinity purified material was done using polyclonal antibodies raised against Sec18p. The presence of this Sec18p suggests that the tagged version of Sec18p is interacting with the wild-type protein possibly within mixed tetramers which can further interact in the proposed fusogenic complex. Unfortunately the presence of Sec17p could not be detected within the same material.

Figure 3.1 Insertion of an *FspI* site into the *SEC18* gene by site directed mutagenesis

The complete ORF of the *SEC18* gene was cloned as a *ScaI/HindIII* fragment from the plasmid pSEY8 into the vector pK18 (digested with *HincII/HindIII*). The *SEC18* gene could then be subcloned into the phage vector M13mp18 as a *BamHI/HindIII* fragment to give the plasmid M13*SEC18* . The oligonucleotide 5'-AATTATGCGCA TTGGGTCA-3' containing a single base substitution was used to anneal to the ssDNA derived from the phage M13*SEC18* and incorporate a new *FspI* site to give the plasmid M13*SEC18*(*FspI*). The introduction of this site was confirmed by restriction enzyme analysis of the double stranded replicative form of DNA and by single-stranded DNA sequencing.

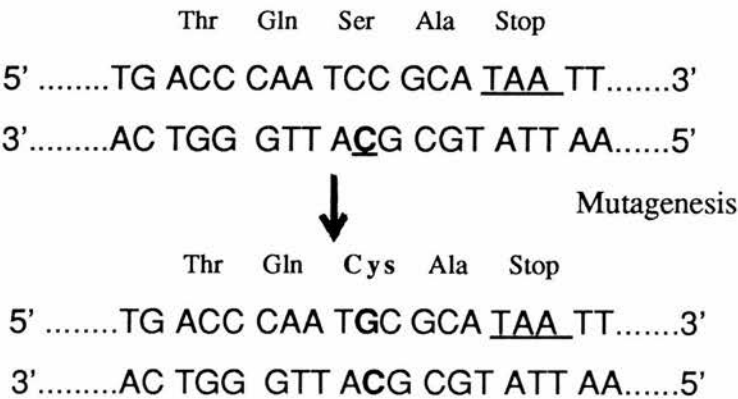


Table 3.1 Complementation of the *sec18-1* mutation by a *Sec18pra* fusion protein. Yeast strains were patched and grown on agar plates on the appropriate selective medium. Patches were replica plated onto glucose and galactose selective medium and grown at 25°C and 37°C and growth monitored visually. '√' indicates strong growth of the yeast cells. 'X' indicates no growth of yeast cells and 'slight' indicates weak growth of yeast cells.

STRAIN	25°C	37°C	
	Growth on glucose media	Growth on glucose media	Growth on galactose media
CHY01	√	X	X
CHY01(pCH1)	√	slight	slight
CHY01(pCH2)	√	slight	slight
CHY01(pCH3)	√	slight	√
CHY01(pCH4)	√	slight	√

Figure 3.2 Fractionation profile of Sec18pra fusion protein in the presence / absence of ATP

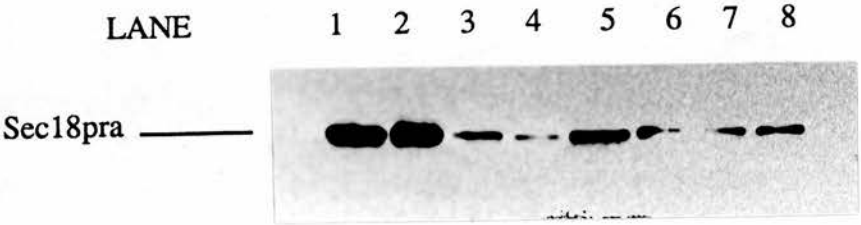
Fractionation analysis was carried out on the yeast strain JRY188 transformed with plasmids pCH3 and pCH4 in the presence or absence of ATP and called JRY188(pCH3) and JRY188(pCH4). Yeast cultures were grown overnight to an OD₆₀₀ of 0.8 then the cells were harvested, washed once with 0.1 M Tris.HCl pH7.4 then resuspended in an appropriate volume of lysis buffer +/- ATP (5 mM ATP, 5 mM MgCl₂) and lysed using glass beads. The crude yeast lysate was centrifuged at 3,000 x g (P3) to remove any unbroken cells and the supernatant was pelleted sequentially at 12,000 x g (P12) and 100,000 x g (P100,S100). A sample of each fraction was run on a 12% SDS PAGE and the presence of the fusion protein within each fraction detected by Western blot analysis using a rabbit anti-bovine IgG HRP conjugate at a 1:10,000 dilution for 20 min. Detection was carried out on this blot using Enhanced Chemiluminescence (ECL).

(a) LANES 1 to 4 contain the fractions P3, P12, P100, S100 respectively from the yeast strain JRY188(pCH3) prepared in the absence of ATP. LANES 5 to 8 contain the identical samples prepared in the presence of ATP.

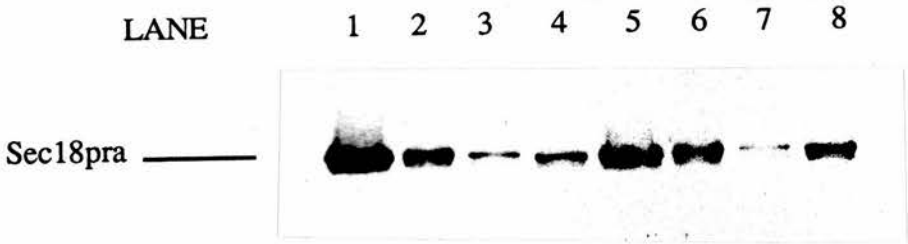
(b) LANES 1 to 4 contain the fractions P3, P12, S100, P100 respectively from the yeast strain JRY188(pCH4) prepared in the absence of ATP. LANES 5 to 8 contain the identical samples prepared in the presence of ATP.

(c) To resolve the two isoforms of the Sec18pra fusion the P100/S100 fractions were analysed on a 7.5% SDS PAGE. LANES 1 to 3 show the P100, S100, and P100/S100 mixed samples from JRY188(pCH3) whereas LANES 4 to 6 contain identical samples prepared from the yeast strain JRY188(pCH4).

(a)



(b)



(c)

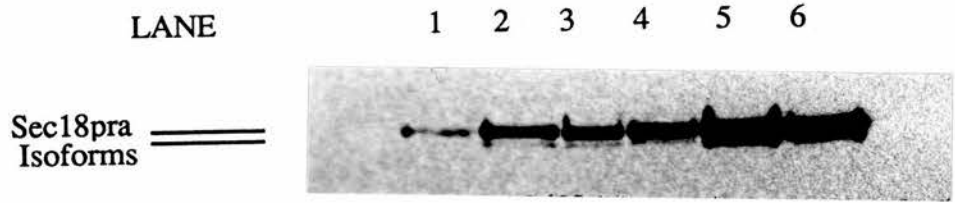


Figure 3.3 Fractionation profile of the Sec18pra fusion protein under various conditions

Yeast strain JRY188(pCH3) was grown in galactose selective medium to an OD₆₀₀ of 0.8. The culture was harvested and lysed using glass beads. The P12 and S12 fractions were prepared using (a) normal lysis buffer (b) lysis buffer containing 0.1 M NaHCO₃ at pH12.5 or (c) lysis buffer containing 2.5 M urea. The S12 fractions were then subjected to further fractionation by differential centrifugation giving a high speed membrane pellet (P100) and supernatant (S100). The distribution of the fusion protein under these conditions was analysed by a 10% SDS PAGE followed by Western blot analysis. The fusion protein was detected using a rabbit anti-bovine IgG HRP conjugate antibody at a 1:10,000 dilution. The Western blot was developed using ECL.

LANE 1 : P100/S100 fractions under normal lysis conditions

LANE 2 : P100/S100 fractions from 0.1 M NaHCO₃ treated extracts

LANE 3 : P100/S100 fractions from 2.5 M urea treated extracts

LANE

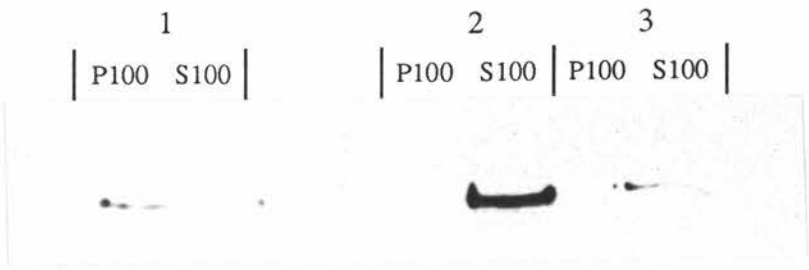


Figure 3.4 Affinity purification of Sec18pra fusion protein from yeast in the presence of an ATP regenerating system

The yeast strains JRY188(pCH3) and JRY188(pCH5) were grown in SGtrp⁻ selective medium at 30°C to an O.D₆₀₀ of 0.8. The cells were harvested and split into two equal samples which were resuspended in 250µl of Buffer A (200 mM Tris.HCl pH7.4; 2 mM EDTA pH8.0; 4 mM PMSF; 200µgml⁻¹ pepstatin, antipain, leupeptin and chymostatin and an ATP regenerating system (1 mM ATP pH7.0 (with NaOH), 8 mM creatine phosphate, 40µgml⁻¹ phosphocreatine kinase) or Buffer B(as Buffer A but without the ATP regenerating system). Samples were lysed using glass beads and centrifuged at 12,000xg for 15 min and the supernatant retained (S12). These extracts were added to 0.7ml of IgG Sepharose (dry volume) which had been prewashed in TST buffer (50 mM Tris.HCl pH7.4; 150 mM NaCl and 0.05% Tween 20) and were then incubated at 4°C for 2hr on a rotating wheel. The material was then transferred into a 'Poly-Prep' column and unbound material was removed by washing with 10X column volume of TST buffer until the A₂₈₀ of the unbound wash was zero. Elution of the fusion protein and Pra was done by adding 2ml of 0.5 M acetic acid pH3.4 to the column. This eluted material was dialysed against distilled water, freeze dried overnight then resuspended in 30µl of water and stored at -70°C. 10µl of each sample was loaded onto two 10% SDS PAGE and either (a) silver stained or (b) analysed by Western blot .

(a) LANE 1 Sec18pra binding in the absence of an ATP regenerating system.

LANE 2 Sec18pra binding in the presence of an ATP regenerating system.

LANE 3 Pra control binding in the absence of an ATP regenerating system.

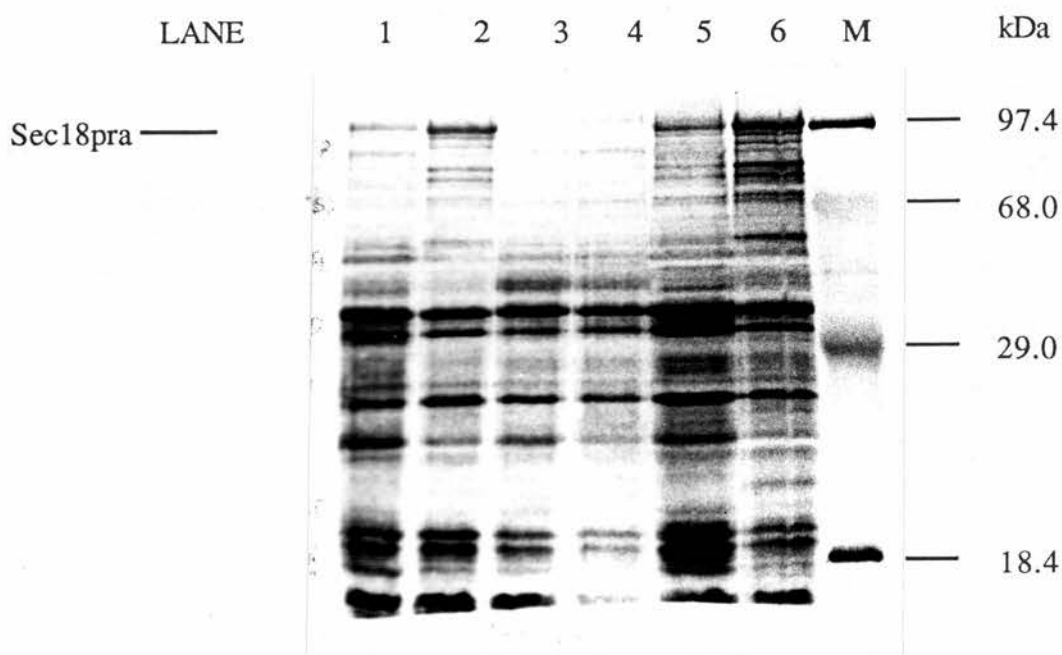
LANE 4 Pra control binding in the presence of an ATP regenerating system.

LANE 5 Mixture of samples loaded in lanes 1 and 3.

LANE 6 Mixture of samples loaded in lanes 2 and 4.

(b) The lanes are exactly the same as those in (a) but proteins were analysed by Western blot analysis, using a rabbit anti-bovine IgG peroxidase conjugate.

(a)



(b)

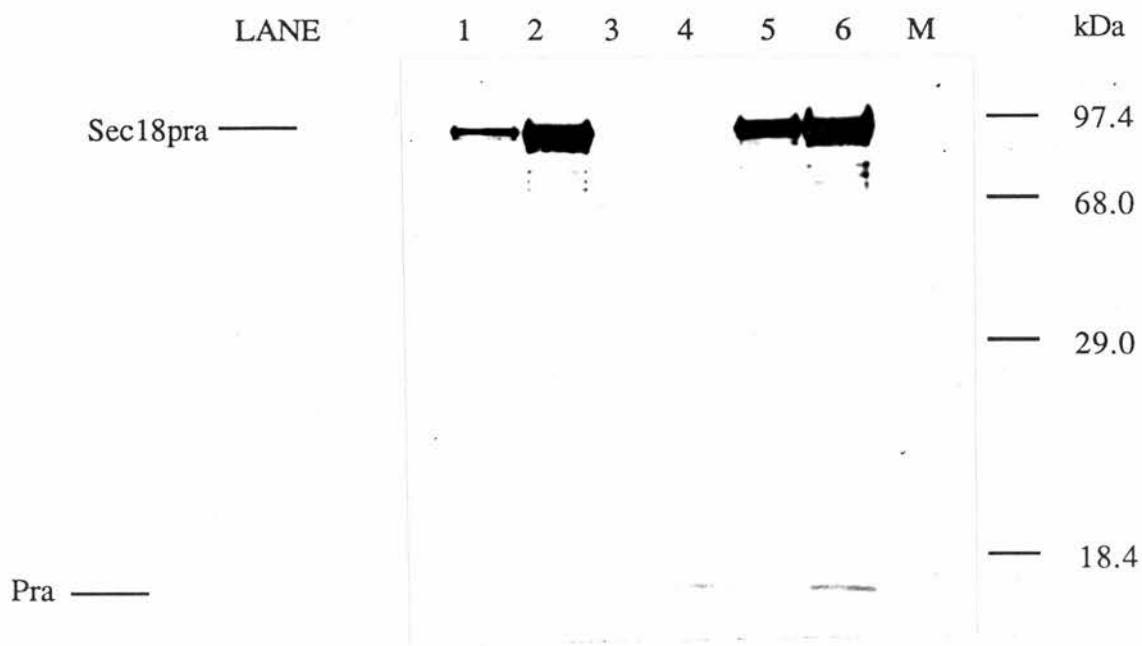


Figure 3.5 Affinity purification of the Sec18pra fusion protein from yeast in the presence of ATP without any magnesium

Yeast strains JRY188(pCH3) and JRY188(pCH5) were grown overnight at 30°C in 600 ml of SGtrp⁻ selective medium to a final O.D₆₀₀ of 0.8. Cells were harvested, washed once in 0.1 M Tris.HCl pH7.4 and resuspended in a modified lysis buffer: 25 mM Tris.HCl pH7.4; 0.75% (w/v) Triton X100; 75 mM KCl; 2 mM EDTA; 0.5 mM ATP; 1% polyethylene glycol (PEG) 4000; 200 µgml⁻¹ each of pepstatin, leupeptin, antipain and chymostatin and 4 mM PMSF. They were then lysed using glass beads and a S12 extract was prepared. Extracts containing half the original cells were added to 0.7ml of IgG Sepharose (dry volume) previously washed in the same lysis buffer and incubated at 4°C for 2 hrs on a rotating wheel. This material was then transferred into a 'Poly-Prep' column which was washed to remove any non-specifically bound proteins with 10X column volume of wash buffer: 20 mM Tris.HCl pH7.4; 0.5% (w/v) Triton X100; 100 mM KCl; 2 mM EDTA and 0.5 mM ATP. Specific complexes were disassembled using 5 ml of elution buffer: Wash buffer plus 8 mM MgCl₂. Both the fusion protein and the Pra control were then eluted from the column using 5 ml of 0.2 M Glycine pH 2.8. The samples eluted from the column using the elution buffer and the low pH buffer were freeze dried overnight and resuspended in 200 µl of distilled water and stored at -70°C. 15 µl of each sample was analysed by SDS PAGE on a 12% gel as shown in

(a) LANE 1: Elution buffer eluant from Sec18pra column.

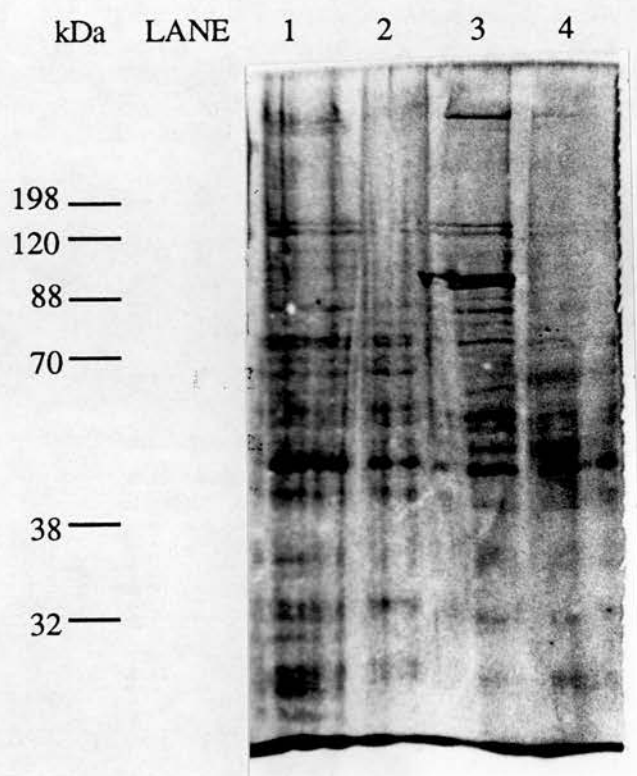
LANE 2: Elution buffer eluant from Pra control column.

LANE 3: Low pH buffer eluant from Sec18pra column.

LANE 4: Low pH buffer eluant from Pra control column.

(b) The other half of the gel containing samples from the same eluants in LANES 5 to 8 were transferred onto a nitrocellulose membrane for Western blot analysis. The presence of pra was detected using the primary antibody a rabbit anti-bovine IgG peroxidase conjugate as previously at a 1: 10,000 dilution.

(a)



(b)

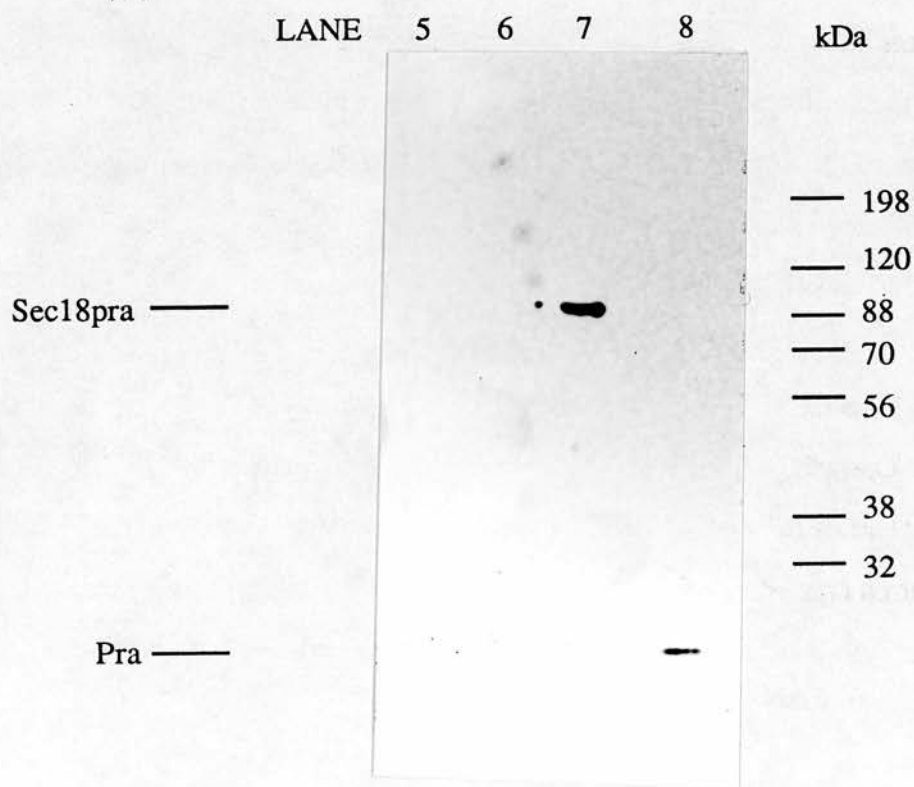
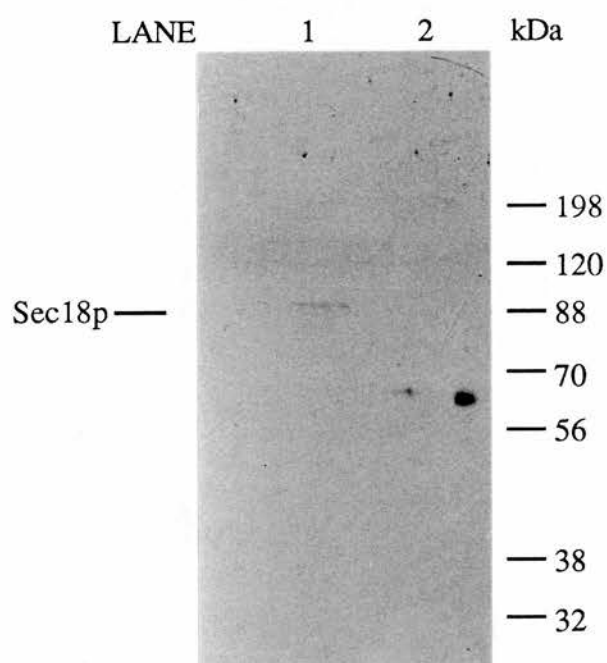


Figure 3.6 Analysis of bound material for presence of the wild-type Sec18p and Sec17p

The Mg⁺⁺ eluants from both the Sec18pra and Pra affinity columns were analysed further by Western blot for the presence of Sec18p and Sec17p. 15µl of the samples were loaded on 12% SDS polyacrylamide gels and transferred to nitrocellulose. These membranes were probed with an affinity purified Sec18p or Sec17p polyclonal antibody in an overnight incubation with a dilution of the primary antibody of 1:100. Detection of this signal using ECL was performed after the membranes had been incubated for 20 min at a 1:5,000 dilution of a second donkey anti-rabbit IgG peroxidase conjugate antibody. The Western blot shown is that which was incubated with the Sec18p specific polyclonal antibody as no Sec17p could be detected in the Mg⁺⁺ eluants.

LANE 1: Elution buffer eluant from Sec18pra column.

LANE 2: Elution buffer eluant from Pra control column.



Appendix to Chapter 3
Production of affinity purified polyclonal
antibodies

Appendix to Chapter 3

3.A.1 Production of affinity purified antibodies against Sec18p and Sec17p

During the course of this work polyclonal antibodies were raised against both Sec18p and Sec17p. Both of these antibodies were used for identification of these proteins in the affinity purified material from yeast using the Sec18pra fusion protein to see if the correct complex was being isolated (Chapter 3). In both cases a fusion between the gene encoding Protein A from *S. aureus* and either the *SEC18* gene or the *SEC17* gene was constructed in the plasmids pCH18 and pCH20 respectively. These fusion constructs were then expressed and purified by affinity chromatography from bacterial cells using Fast Flow IgG Sepharose and used for the immunization of rabbits (see Materials and Methods). Protein A fusions have been reported to give good immune responses (Lowenadler *et al.*, 1986), possibly due to the repetitive globular units of the protein A moiety.

Affinity purification of specific Sec18p and Sec17p antibodies was carried out as described in Materials and Methods using an analogous second set of fusions between the gene encoding β -galactosidase and either the *SEC18* gene (pCH19) or the *SEC17* gene (pCH21). Affinity purified Sec18p and Sec17p antibodies were shown to recognise proteins of the correct molecular size of 82 kDa and 84 kDa in the case of Sec18p and 33 kDa in the case of Sec17p in lysates prepared from both wild-type and over-expressing yeast strains as shown in **Figure 3.A.1** and **Figure 3.A.2**. Two major degradation products are observed for the wild-type Sec18p which are not seen in the case of the Sec18pra fusion protein used in Chapter 3. It may be that the Pra tag of the fusion protein somehow stabilises the Sec18p *in vivo* possibly protecting Sec18p from C-terminal degradation. As can be seen from **Figure 3.A.2** the titre of the Sec17p antibody is much lower than that observed for the Sec18p antibody.

Figure 3.A.1 Detection of Sec18p by affinity purified polyclonal antibodies.

A low speed supernatant (S12) was prepared from the wild-type yeast strain JRY188, and the transformed strain JRY188(pCH7), which has the *SEC18* gene under the control of the *GALI* promoter, after growth on galactose medium and fractionated by SDS PAGE on a 10% gel. The proteins were transferred to nitrocellulose and the blot was analysed by Western analysis using, as first antibody, affinity purified anti-Sec18p antibodies (see Materials and Methods for production) at a 1 : 100 dilution overnight. The second antibody was a donkey anti-rabbit IgG-HRP conjugate used at a 1 : 5,000 dilution for 20min and was detected was using ECL.

LANE 1 : S12 extract from wild-type strain JRY188.

LANE 2 : S12 extract from yeast strain JRY188(pCH7).

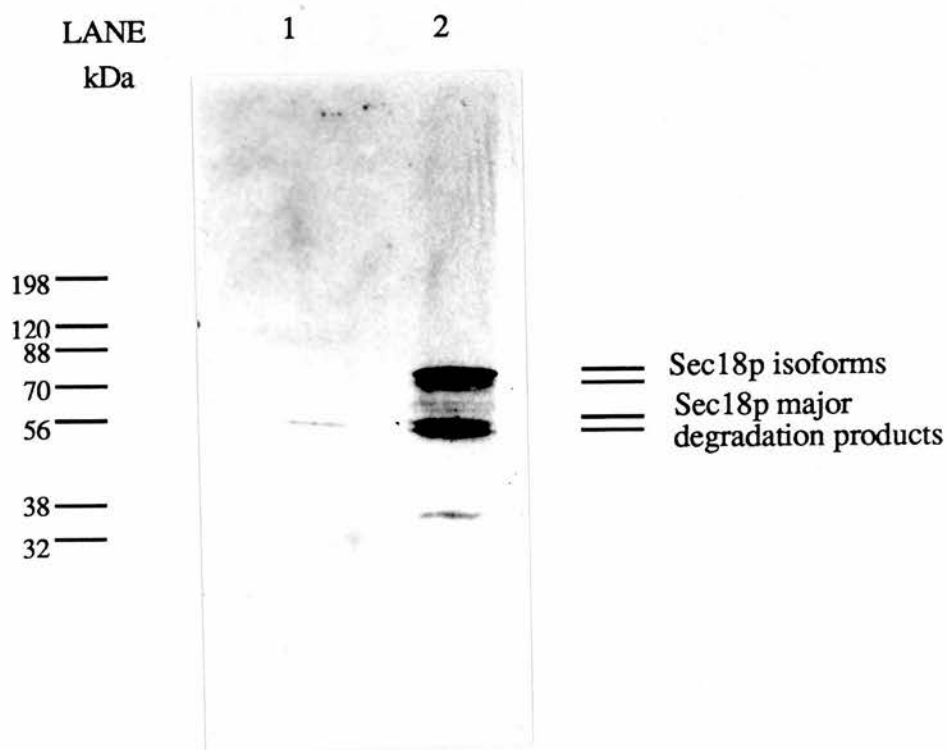


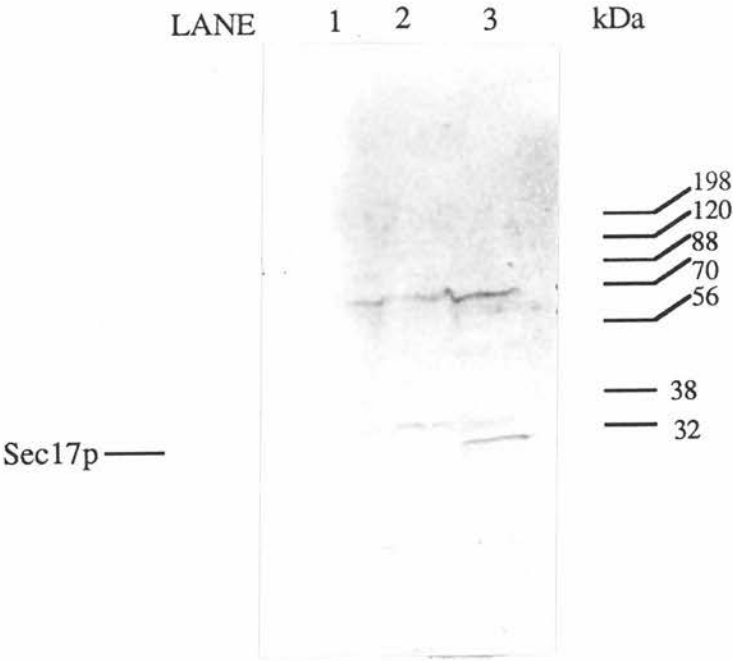
Figure 3.A.2 Detection of Sec17p by affinity purified polyclonal antibodies.

A low speed supernatant (S12) was prepared from the wild-type yeast strain JRY188, and the transformed strains JRY188(pCH22) and JRY188(pCH22A) which are expected to have low and high expression levels of the Sec17p respectively. Samples were fractionated by SDS PAGE on a 15% gel, the proteins were transferred to nitrocellulose and the blot was analysed by Western analysis using, as first antibody, affinity purified anti-Sec17p antibodies (see Materials and Methods for production) at a 1 : 100 dilution overnight. The second antibody was a donkey anti-rabbit IgG-HRP conjugate used at a 1 : 5,000 dilution for 20min and was detected using ECL.

LANE 1 : S12 extract from wild-type strain JRY188.

LANE 2 : S12 extract from yeast strain JRY188(pCH22).

LANE 3 : S12 extract from yeast strain JRY188(pCH22A).



Chapter 4

***Random mutagenesis of the SEC18 gene and
isolation of dominant negative alleles of this gene***

4.1 Introduction

A number of genetic strategies have been successfully applied within yeast in an attempt to elucidate the steps of vesicle mediated transport. The isolation of yeast mutants defective in protein secretion (*sec* mutants), retention of soluble ER proteins (*erd* mutants), or sorting of vacuolar proteins (*vps* mutants) has allowed the identification of a number of genes which act at different stages of the secretory pathway (Novick *et al.*, 1980, Pelham *et al.*, 1988, Banta *et al.*, 1988). A functional relationship between proteins may be indicated when two defective mutants have a lethal phenotype as a double mutant showing synthetic lethality, or the genetic defect of one mutant is suppressed by another gene. No extragenic suppressors of the original *sec18-1* temperature sensitive allele have been isolated (T. Graham, personal communication). This chapter introduces an alternative genetic strategy taken to identify novel proteins that may interact with the *SEC18* gene product.

The *SEC18* gene encodes two gene products of approximately 82 kDa and 84 kDa (Eakle *et al.*, 1988). These polypeptides are thought to form an active tetramer involved in vesicle transport. A dominant negative mutant (DNM) form of the *SEC18* gene will encode mutant Sec18p monomers which should interact with and inhibit the normal function of the wild-type protein *in vivo* in a number of ways (Herskowitz, 1987). Mutant forms of Sec18p could show 'internal dominance' by forming mixed tetramers with the wild-type polypeptide giving non-functional tetramers that cannot interact with other 'target' proteins during normal Sec18p function. 'External dominance' would result when potentially active mixed tetramers interact with specific target proteins but in a non-functional way by forming either unstable fusion complexes or complexes that are locked into a non-functional form. Alternatively, the aberrant Sec18p tetramer could lose the normal specificity observed for fusion with a target vesicle and so promote deleterious targeting / fusion events within the yeast cell. This chapter gives a description of the strategies used to mutate

the *SEC18* gene and screen for genes encoding DNM forms of the Sec18p. An initial analysis of two of the mutants shows the extent to which the *SEC18* gene in each case was mutagenised.

4.2 Insertion of the SEC18 gene downstream of the GAL1 promoter within yeast expression vectors

To regulate the expression of Sec18p within yeast cells the gene was first cloned into a low copy yeast expression vector YCpGAL. The *SEC18* gene was cloned as a 3.0 kbp *Bam*HI/*Hind*III fragment from the vector pSEY8 into the *Bam*HI/*Hind*III sites of YCpGAL to produce the construct pCH7. The *SEC18* gene was inserted downstream of the strong *GAL1* promoter allowing the expression of deleterious gene products to be conditional on the presence of galactose and repressed by the presence of glucose in the growth medium.

4.3 Random mutagenesis of the SEC18 gene and screening for dominant negative mutations within the SEC18 gene

Since the sites of interaction between Sec18p and other proteins within the proposed fusion complex have not been characterised a random mutagenic approach was adopted. Two strategies were used; *in vitro* polymerase chain reaction (PCR) mutagenesis and *in vivo* mutagenesis using a *mutD* strain of *E. coli*. PCR mutagenesis is based upon a normal reaction carried out under sub-optimal conditions with respect to cation concentrations and a bias in nucleotide concentrations (see Materials and Methods). This decreases the fidelity of replication by *Taq* polymerase during amplification of the DNA fragment. The *mutD* strain of *E. coli* contains a mutated ϵ subunit of DNA polymerase III affecting the 3' \rightarrow 5' exonuclease activity of this enzyme and so interfering in the proof-reading ability within the bacterial strain during DNA replication (see Materials and Methods).

The amplified DNA resulting from the mutagenic PCR reaction was cotransformed with a gapped yeast expression vector (YCpGAL) into an appropriate yeast strain. This resulted in the insertion of the PCR products into the gapped plasmid by homologous recombination events *in vivo* (as shown in **Figure 4.1.**) and allowed the direct screening for mutants within the yeast strain. The *mutD* mutagenesis however produced a mutagenised pool of plasmid DNA which had to be transformed into the appropriate yeast strain (JRY188 or CHY01) for screening purposes. In both cases individual yeast transformants were isolated and screened by replica-plating of patched colonies onto various growth media at different temperatures.

The yeast strain CHY01 which contains the temperature sensitive *sec18-1* allele was used to measure the amount of mutagenesis incurred under the three mutagenic conditions. This strain does not grow at the nonpermissive temperature of 37°C. When transformed with DNA from a mutagenic reaction the transformants will either grow at 37°C on medium containing galactose due to production of a wild-type gene product which complements the ts defect, or will show no growth under these induced conditions if an inactive mutant gene product is being expressed. As seen in **Table 4.1.** a high proportion of ts phenotype is observed when using PCR mutagenesis compared to the *mutD* strategy.

As described previously, a DNM form of the Sec18p should interact in a negative way with wild-type Sec18p when induced by the presence of galactose as a carbon source in the growth medium. This interaction should affect the ability of the wild-type Sec18p to participate in membrane fusion/docking events occurring throughout the secretory pathway. As such, the growth of the transformed yeast cells is expected to be normal in glucose media but should be decreased in galactose media due to the induced DNM form of Sec18p interfering with the wild-type polypeptide function. The yeast strain JRY188 which is *SEC18*⁺ was used to screen for the DNM

phenotype (**Figure 4.2.**).

A total of 3000 transformed colonies resulting from experiments using three methods were screened giving an initial pool of 32 potential DNM forms of the *SEC18* gene, however on rechecking of the phenotype and selection for absolute galactose-sensitivity the pool was restricted to 5 showing a plasmid dependent DNM phenotype (see **Table 4.1.**). The plasmids were recovered from galactose-sensitive transformants by preparing total DNA from the 5 individual colonies. This DNA was then transformed into the *E. coli* strain NM522, and the plasmids which represent 5 DNM forms of the *SEC18* gene were recovered; 2 originating from the *mutD* mutagenesis and 3 from the PCR mutagenesis. The mutagenised *SEC18* gene was cloned from these plasmids in each case as a *Bam*HI/*Hind*III cassette into a new YCpGAL yeast expression vector and the phenotype rechecked in the yeast strain JRY188. The constructs were named pCH8, 9, 10, 11 and pCH12 and contained *SEC18-108*, *109*, *110*, *111* and *112* DNM alleles respectively.

4.4 Analysis of mutations within the *SEC18* gene

To get an idea as to the extent of mutagenesis obtained by both strategies two of the isolates were sequenced, one originating from the *mut D* mutagenesis (*SEC18-109*) and the other from PCR mutagenesis (*SEC18-112*). These particular alleles were chosen because the phenotype of yeast strains harbouring them has been characterized extensively and has been shown to be very different (see Chapter 5). Since the gene products produced from these alleles have such different effects *in vivo* sequence analysis may give some information as to the important residues that are responsible for the dominant negative phenotype seen *in vivo*.

The sequencing strategy involved a number of oligonucleotides specific to the wild-type *SEC18* gene as shown in **Figure 4.3.** The sequences of the two mutants

(*SEC18-109* and *SEC18-112*) were compared with that obtained on resequencing the wild-type *SEC18* gene which had been used as the original template for mutagenesis. On resequencing this wild-type *SEC18* gene an extra codon (GTG) was identified between the original codons GAT₁₆₇₉ and GTT₁₆₈₂ as defined in the *SEC18* gene sequence cloned by Eakle *et al.*, 1988. This extra codon identified within this work as GTG₁₆₈₂ encodes an additional glycine residue in the amino acid sequence of Sec18p (see **Figure 4.3.**). This Gly residue is predicted to be present in both the recently identified Sec18p from *Candida albicans* (Nieto *et al.*, 1993) and the mammalian NSF protein (Wilson *et al.*, 1989) and another group has recently resequenced the *SEC18* gene as part of the yeast genome project and submitted the revised sequence to the EMBL database library (Van der Aart *et al.*, 1994).

The type and number of mutations observed in these two mutants is summarised in **Table 4.2.** PCR mutagenesis has mutated the *SEC18* gene to the greatest extent: The type of base changes shown in the *SEC18-112* allele mainly being A/T, the most abundant of which are A/T → G/C transitions. This has resulted in 0.5% mutagenesis of the ORF of the *SEC18* gene although all of these mutations are not relevant at the amino acid level and therefore only 1% of the Sec18p is in fact mutagenised by this method. The *mutD* strategy however shows a much lower degree of mutations in the *SEC18-109* allele of 0.04% at the nucleotide level and 0.1% at the amino acid level. It is worth noting that the mutations seen in both mutant alleles do not occur within the proposed ATP binding sites of the *SEC18* gene.

4.5 Further analysis of the *SEC18-112* mutant allele

Although twelve mutations were found within the *SEC18-112* mutant only eight of these are relevant at the amino acid level and therefore could be responsible for the DNM phenotype observed within strains harbouring this allele. To try and identify the residues which are important in retaining the wild-type Sec18p phenotype and may

contribute to the DNM phenotype when mutated, a 'hybrid domain' strategy was adopted. As shown in **Figure 4.4(a)** the *SEC18-112* allele can be divided into three regions (N region, M region and C region) which contain a number of relevant mutations, using restriction endonucleases. The positions of each restriction endonuclease are as shown in **Figure 4.3**. This allows chimaeric proteins to be made by cloning the wild-type *SEC18* sequence in to replace each domain separately producing hybrids that can then be expressed within the yeast strain JRY188 and the phenotype analysed. It is shown in **Figure 4.4(b)** that on replacing the C region of *SEC18-112* with a wild-type *SEC18* sequence the gene still encodes a protein which has a DNM phenotype on galactose medium, whereas replacing either the N or M region results in the expression of a protein that does not exhibit a DNM phenotype. From these initial observations it seems that the three mutations within the C region of the *SEC18-112* allele are not responsible for the DNM phenotype seen within strains harbouring this allele. Thus the mutations responsible for the DNM phenotype may be localised to six mutations within the N and M regions of the gene but at this point it is not known if all of these mutations are relevant or contribute equally to the DNM phenotype observed.

4.6 Further analysis of the *SEC18-109* mutant allele

Only one mutation was observed within this mutant allele resulting in the alteration of Thr₃₉₄ to Pro and the expression of a DNM phenotype on galactose medium. This mutation does not occur in either of the proposed ATP binding sites within the Sec18p sequence (**Figure 4.3**). The structure of the Sec18p, and therefore the regions which are in contact with other proteins within the proposed fusion complex, is as yet unknown, however as shown in **Figure 4.5** the altered Thr₃₉₄ residue is within a highly conserved region between NSF, Sec18p from *Candida albicans* and the Sec18p from *S. cerevisiae* suggesting that this residue may be important either structurally or functionally within the protein. Does this amino acid change alter the predicted

secondary structure of the Sec18p? Using a secondary-structure prediction based on the combination of several separate methods (Sawyer *et al.*, 1986) it can be seen that there is no obvious alteration to the structure at this level and a β -strand is predicted within this region for both the wild-type and mutant protein (as shown in **Figure 4.6**).

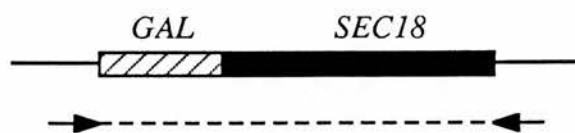
4.7 Conclusion

Within this Chapter I have described two methods of random mutagenesis which I have used to mutate the *SEC18* gene of *S. cerevisiae* and identify 5 DNM mutant alleles of this gene (*SEC18-108,109, 110, 111* and *112*). Sequence analysis of the *SEC18* gene revealed an extra codon which was not identified within the original sequence. Two mutant alleles were also sequenced; *SEC18-109* (resulting from *mutD* mutagenesis) and *SEC18-112* (resulting from PCR mutagenesis). *SEC18-109* contains one mutation which results in a single amino acid change of a highly conserved Thr residue which results in a mutant Sec18p that is responsible for the observed DNM phenotype *in vivo*. The exact role of this residue within the structure of Sec18p is not yet known although initial studies show that this residue does not change the secondary structure of the protein significantly. *SEC18-112* however contains eight mutations at the amino acid level, and initial analysis has shown that the first six of these mutations may be responsible for the DNM phenotype of this protein *in vivo*.

Figure 4.1 Cotransformation of muutagenised SEC18 gene and a gapped plasmid into the yeast strain JRY188.

The oligonucleotides 5'- GCTCACTCATTAGGCACC -3' and 5'-CCGCACA GATGCGTAAGG -3' were used to anneal to pCH7 and produce a PCR product of approximately 4.1 kbp containing the *GALSEC18* cassette. The mutagenic PCR reaction conditions were first optimised for MgCl_2 concentration at 5 mM. Either 0.4 mM or 0.6 mM MnCl_2 was used giving a ratio of 8 : 1 or 12 : 1 of Mg^{++} to Mn^{++} ions respectively in the PCR reaction. The resultant PCR reaction mixture contains a population of wild-type and mutagenised DNA fragments.

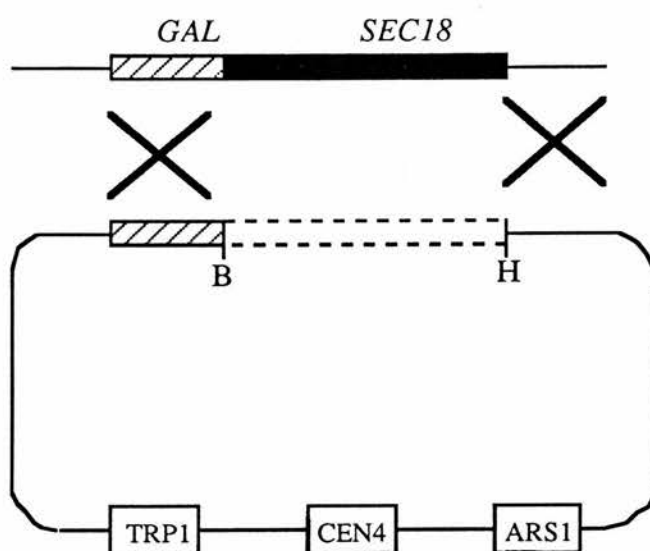
This reaction mixture was cotransformed with the plasmid YCpGAL (gapped with *Bam*HI/*Hind*III) into the yeast strain JRY188. Homologous recombination *in vivo* repairs the plasmid with the PCR product thus producing yeast transformants carrying individual plasmids expressing a wild-type or mutant form of the Sec18p.



I. Mutagenise GALSEC18 by PCR



2. Recombine PCR product with gapped plasmid in vivo



3. Screen Trp^+ transformants for dominant negative phenotype.

Table 4.1 *Extent of mutagenic phenotype observed for three random mutagenic strategies.*

Method of mutagenesis	Number of colonies screened	% of CHY01 transformants not able to complement the <i>sec18-1</i> defect at 37°C	% of JRY188 transformants not able to grow on galactose medium at 25°C
Propagation in the <i>E.coli</i> strain <i>mutD</i>	2867	4.8	0.8
PCR 12 : 1	56	45	16
8 : 1	77	22	3

Figure 4.2. Screening for a dominant negative Sec18p phenotype in the yeast strain JRY188.

The yeast strain JRY188 was cotransformed with (a) PCR amplified DNA and a gapped YCpGAL plasmid (as described in **Figure 4.1.**) or (b) transformed with a mutagenised DNA pool obtained from the *mutD* mutagenesis (see Materials and Methods). The resultant Trp⁺ transformants were plated out onto SDTrp⁻ (glucose) plates and grown at 25°C. Individual colonies were picked and patched onto fresh SDTrp⁻ plates at 25°C. These yeast patches were replica-plated onto both glucose (SD) and galactose (SG) selective plates and growth was monitored at 25°C and compared to patches of the yeast strain JRY188(pCH7) which expresses the wild-type Sec18p when grown on galactose medium.

JRY188 transformed with
mutagenised DNA



Patched colonies on SD Trp⁻
medium

Replica - plate



STRAIN

Growth on SDTrp⁻
media at 25°C

Growth on SGTrp⁻
media at 25°C

Wild-type yeast
JRY188

√

√

JRY188 transformed
with mutagenised DNA

√

X

Figure 4.3 Revised sequence of the *SEC18* gene and the predicted amino acid sequence

The wild-type *SEC18* gene and two mutant alleles (*SEC18-109* and *SEC18-112*) carried on plasmids pCH7, pCH9 and pCH12 respectively were sequenced using standard double stranded plasmid sequencing (see Materials and Methods) using *SEC18*-specific primers corresponding to the + strand of DNA. The direction of the oligonucleotides are shown as single arrows and only one strand was sequenced. An additional codon encoding a Gly residue was identified and is highlighted in bold and the two consensus sequences for ATP binding are boxed. The position of the restriction sites: *Bam*HI [GGATCC]; *Bsm*I [GAATGCC]; *Nru*I [TCGCGA] and *Hind*III [AAGCTT] are underlined. The numbering of the nucleotide and amino acid sequences were subsequently modified from that of the original published *SEC18* sequence (Eakle *et al.*, 1988).

Arg Phe Glu Val Gln Val Glu Ile His Leu Pro Asp Glu Lys Gly Arg Leu 425
 1766 AGA TTT GAA GTC CAA GTT GAA ATT CAT TTA CCC GAT GAA AAA GGA AGA CTC
 Gln Ile Phe Asp Ile Gln Thr Lys Lys Met Arg Glu Asn Asn Met Met Ser 442
 1817 CAA ATT TTC GAC ATT CAG ACG AAG AAA ATG AGG GAA AAT AAT ATG ATG AGC
 Asp Asp Val Asn Leu Ala Glu Leu Ala Ala Leu Thr Lys Asn Phe Ser Gly 459
 1868 GAC GAT GTT AAC TTA GCT GAG TTA GCT GCG TTA ACA AAA AAC TTC TCT GGT
 Ala Glu Ile Glu Gly Leu Val Lys Ser Ala Ser Ser Phe Ala Ile Asn Lys 476
 1919 GCT GAG ATT GAG GGT TTA GTG AAG AGT GCA AGT TCT TTT GCA ATC AAC AAA
 Thr Val Asn Ile Gly Lys Gly Ala Thr Lys Leu Asn Thr Lys Asp Ile Ala 493
 1970 ACC GTC AAC ATC GGG AAA GGT GCC ACA AAA CTT AAC ACT AAA GAT ATA GCA
 Lys Leu Lys Val Thr Arg Glu Asp Phe Leu Asn Ala Leu Asn Asp Val Thr 510
 2021 AAA CTT AAA GTA ACA AGA GAA GAC TTT TTA AAT GCA CTC AAC GAT GTT ACT
 Pro Ala Phe Gly Ile Ser Glu Glu Asp Leu Lys Thr Cys Val Glu Gly Gly 527
 2072 CCC GCT TTT GGG ATT AGT GAA GAA GAT TTG AAA ACA TGT GTG GAA GGT GGA
 Met Met Leu Tyr Ser Glu Arg Val Asn Ser Ile Leu Lys Asn Gly Ala Arg 544
 2123 ATG ATG CTT TAT TCC GAA CGA GTT AAC TCA ATA TTG AAG AAC GGA GCC CGT
 Tyr Val Arg Gln Val Arg Glu Ser Asp Lys Ser Arg Leu Val Ser Leu Leu 561
 2174 TAC GTC CGC CAA GTT CGC GAG AGT GAT AAA TCC AGG TTA GTA TCT CTA TTA
 Ile His Gly Pro Ala Gly Ser Gly Lys Thr Ala Leu Ala Ala Glu Ile Ala 578
 2225 ATC CAC GGC CCT GCA GGG TCC GGT AAA ACA GCT TTA GCC GCT GAA ATT GCT
 Leu Lys Ser Gly Phe Pro Phe Ile Arg Leu Ile Ser Pro Asn Glu Leu Ser 595
 2276 TTA AAA TCT GGA TTC CCA TTC ATC AGG TTA ATT TCT CCC AAC GAG TTG TCA
 Gly Met Ser Glu Ser Ala Lys Ile Ala Tyr Ile Asp Asn Thr Phe Arg Asp 612
 2327 GGC ATG TCA GAA AGC GCA AAA ATT GCC TAT ATT GAT AAC ACT TTC AGA GAT
 Ala Tyr Lys Ser Pro Leu Asn Ile Leu Val Ile Asp Ser Leu Glu Thr Leu 629
 2378 GCG TAT AAA TCT CCA CTA AAC ATT CTT GTT ATT GAT TCG TTA GAG ACT CTA
 Val Asp Trp Val Pro Ile Gly Pro Arg Phe Ser Asn Asn Ile Leu Gln Met 646
 2429 GTT GAT TGG GTA CCA ATT GGT CCA AGA TTC TCT AAT AAC ATT TTA CAA ATG
 Leu Lys Val Ala Leu Lys Arg Lys Pro Pro Gln Asp Arg Arg Leu Leu Ile 663
 2480 CTA AAG GTT GCA TTG AAG CGT AAA CCC CCA CAA GAC CGT CGT TTA TTG ATC
 Met Thr Thr Thr Ser Ala Tyr Ser Val Leu Gln Gln Met Asp Ile Leu Ser 680
 2531 ATG ACT ACT ACA TCA GCT TAT TCG GTA CTT CAA CAA ATG GAT ATC TTG AGT
 Cys Phe Asp Asn Glu Ile Ala Val Pro Asn Met Thr Asn Leu Asp Glu Leu 697
 2582 TGC TTC GAC AAT GAG ATA GCA GTT CCA AAT ATG ACC AAT TTA GAT GAA TTG
 Asn Asn Val Met Ile Glu Ser Asn Phe Leu Asp Asp Ala Gly Arg Val Lys 714
 2633 AAC AAC GTC ATG ATA GAA TCA AAC TTT CTT GAC GAT GCT GGT AGA GTT AAA
 Val Ile Asn Glu Leu Ser Arg Ser Cys Pro Asn Phe Asn Val Gly Ile Lys 731
 2684 GTT ATT AAT GAA TTA TCA AGG AGC TGT CCT AAC TTC AAT GTC GGT ATT AAA
 Lys Thr Leu Thr Asn Ile Glu Thr Ala Arg His Asp Glu Asp Pro Val Asn 748
 2735 AAG ACC TTG ACC AAC ATT GAA ACC GCA AGG CAC GAT GAA GAT CCC GTG AAC
 Glu Leu Val Glu Leu Met Thr Gln Ser Ala Stop 758
 2786 GAG CTT GTT GAG TTG ATG ACC CAA TCC GCA TAA TTATTTCAAATTTTTCATGTTCT
 2842 TGTATTTTATTCTTTATCCCTTCCAATCAGAAAGGATCTAGTGAACAAGTTCTTTCCCTCTATGGTA
 2909 TATATTTTAGTGATAAATTTTATAAAATTATAAAACCAAGGCATCCTTTTCTTTTATTCTGTCAATTG
 2976 GATTCTGCCTGTCAATAATTATCACCAACCGGGTAAAGATGATAATTTTTCAGTCGCTTTGCCCGAGAA
 3043 GCTT

Table 4.2 Mutations observed within the SEC18 gene after two different mutagenic strategies

Mutagenic strategy	Nucleotide change	Amino acid change
PCR mutagenesis (SEC18-112)	T ₅₉₆ — C	Met ₁₉ — Thr
	C ₆₁₈ — G	Thr ₂₆ — Ser
	T ₉₄₂ — C	Phe ₁₃₄ — Ser
	A ₁₀₃₀ — G	- SILENT -
	G ₁₆₄₃ — T	Gly ₃₆₈ — Trp
	A ₁₇₆₈ — G	- SILENT -
	A ₂₀₉₄ — G	Glu ₅₁₈ — Gly
	A ₂₁₀₂ — G	Lys ₅₂₁ — Glu
	A ₂₃₁₆ — G	Asn ₅₉₂ — Ser
	A ₂₃₉₂ — G	- SILENT -
	A ₂₆₂₅ — G	Asp ₆₉₅ — Gly
	A ₂₆₉₅ — G	- SILENT -
mutD mutagenesis (SEC18-109)	A ₁₇₂₁ — C	Thr ₃₉₄ — Pro

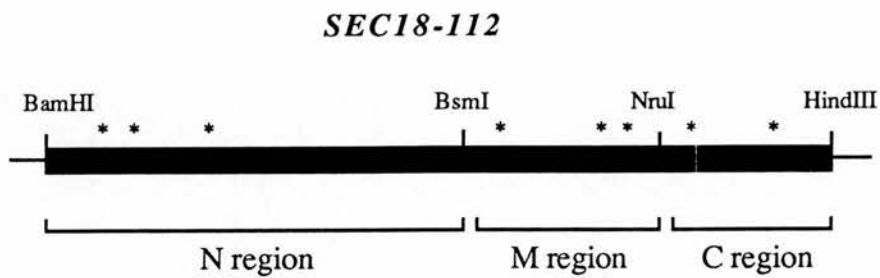
Figure 4.4 Construction and expression of hybrid genes between the *SEC18-112* gene and the wild-type *SEC18* gene

Three hybrid constructs were made by cloning the wild-type *SEC18* sequence from plasmid pCH7 as a *Bam*HI/*Bsm*I (N region), *Bsm*I/*Nru*I (M region) or *Nru*I/*Hind*III (C region) into the corresponding sites within the *SEC18-112* mutant allele carried on the plasmid pCH12. The positions of these restriction sites within the *SEC18* gene are shown in **Figure 4.3**. The resulting constructs were named pCH23, 24 and 25 and were transformed into JRY188. The transformed strains JRY188(pCH23 to 25) were grown in 10 ml of glycerol / ethanol selective medium at 25°C until the culture was in mid-exponential growth. At this point 3% (w/v) galactose was added to the medium and the growth of the cultures was monitored for a further 5 hrs at 25°C.

(a) Schematic diagram showing the positions of the mutations within the three regions of the *SEC18-112* mutant allele.

(b) Graph showing the growth of transformed strains JRY188(pCH23 to 25) in galactose medium over a 5hr time course.

(a)



KEY: * Mutation within nucleotide sequence which is relevant at the amino acid level

(b)

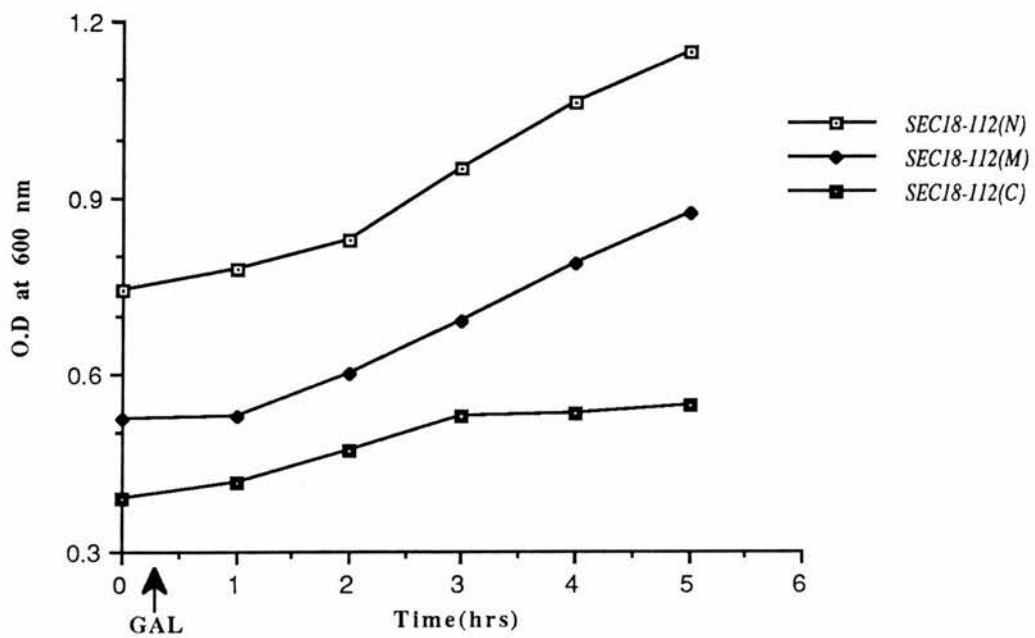


Figure 4.5 Sequence alignment between the *S. cerevisiae* Sec18p, the *C. albicans* Sec18p and mammalian NSF

The amino acid sequence of the Sec18p from *S. cerevisiae* was aligned with that of the Sec18p from *C. albicans* and the mammalian NSF sequence respectively using the program 'pileup' on the 'U.W.G.C.G.' sequence analysis package. The middle sequence shown is that of Sec18p from *S. cerevisiae* and the symbol '.' represents an identical sequence alignment of this sequence with the *C. albicans* Sec18p sequence or the NSF sequence respectively. The mutant allele *SEC18-109* contains a Thr₃₉₄ to Pro mutation which can be seen as the boxed residue within this alignment and occurs in a region which is highly conserved between these three proteins.

C_Sec18p MEKLGFKHVS NSPSSPSQPK ITHNRPIPHP QSPPLPQRPL SEPLKKQLLV DNSPGNDVVI ANCVAVNAQD FQNIPDRAPV

 Sec18p ..MFKIPGFG KAAANHTPPD MTN..... MDTTRTRLKV SNCPNNSYAL ANVAAVSPND F...PNNIYI

 NsfMAGRSMQA ARCPTDELSL SNCVVSEKD YQSQGHVIVR

C_Sec18p ILDG.VFVYS IAKDDRVRPG TIGLAGNMRT WGKWSLGQPV NVENYNIFHN GQQQQYLGA I DLLIDFRAKA RANLNPIHND

 Sec18p IIDN.LFVFT TRHSNDIPPG TIGFNGNQRT WGGWSLNQDV QAKAFDLFKY SGKQSYLGS I DIDISFRARG KAVSTVFDQD

 Nsf TSPNHKIYFT LRTHPSVVP G SVAFSLPQRK WAGLSIGQE I EV...ALYSF DKAKQCIGTM TIEIDFLQKK NIDSNPYDTD

C_Sec18p ELVALFLKNY ENQILQPTQV IYMEYTG IYF QIRVNNVQII DVNTKDQLPS FKSDDDINTK GILIKSTDVG FYPYEGSIIN

 Sec18p ELAKQFVRCY ESQIFSPTQY LIMEFQGHFF DLKIRNVQAI DLGDIE..PT SAVATGIETK GILTKQTQIN FFKGRDGLVN

 Nsf KMAAEFIQQF NNQAFSVGQQ LVFSFNDKLF GLLVKDIEAM DPSILKGEPA SGKRQKIEV. GLVVGNSQVA FEKAENSSLN

C_Sec18p LTKPKTLKQR MFGGSTPHRT SRRKQIINPD FKLEDLIGGG LDAEFQDIFR RAFNSRILPP ELAEKLDYKH CKGLLLYGPP

 Sec18p LKSSNSLR.. ..PRNAVIRPD FKFDLGVGG LDKEFTKIFR RAFASRIFFP SVIEKLGISH VKGLLLYGPP

 Nsf LIGKAKTKE.NRQSIINPD WNFEKMIGG LDKEFSDIFR RAFASRVFPP EIVEQMGCKH VKGILLYGPP

C_Sec18p GTGKTLIARK LSKMLNGKEP KIVNGPEMLS KYVGASEENI RNLFKDAEAE YKLKGEDSDL HVIIFDELDS VFKQRGSGKS

 Sec18p GTGKTLIARK IGTMLNAKEP KIVNGPEILS KYVGSSEENI RNLFKDAEAE YRAKGEESL HIIIFDELDS VFKQRGS.RG

 Nsf GCGKTLARQ IGKMLNAREP KVVNGPEILN KYVGESEANI RKLFADEEEE QRRIGANSGL HIIIFDEIDA ICKQRGS.MA

C_Sec18p DGTGVGDNVV NQLLSKMDGV DQLNNILVIG MINRLDLIDT ALLRPGRFEI QIEISLPDEK GRKDI FLIHT KKL TENGILS

 Sec18p DGTGVGDNVV NQLLAKMDGV DQLNNILVIG MINRKDLIDS ALLRPGRFEV QVEIHLPEK GRLQIFDIQT KKMRENMMMS

 Nsf GSTGVHDTVW NQLLSKIDGV EQLNNILVIG MINRPDLIDE ALLRPGRLEV KMEIGLPDEK GRLQILHIHT ARMGRGHQLS

C_Sec18p SDVNFDELST LTKNFTGAEI EGLCNSAKSY AISRHTKKG. ALAQIDPESI AKMKITRDDF LLAL.NDIRP AFGTDEEDLS

 Sec18p DDVNLAEALAA LTKNFSGAEI EGLVKSASSF AINKTVNIGK GATKLNTKDI AKLKVTREDF LNAL.NDVTP AFGISEEDLK

 Nsf ADVDIKELAV ETKNFSGAEL EGLVRAAQST AMNRHIK.AS TKVEVDEKA ESLQVTRGDF LASLENDIKP AFGTNQEDYA

C_Sec18p QQAQHGIIQF NQTIRNIFEK GQSIIDVVRS SETEHLRSIL LYGPPGVGKT SIATTLALNS DFPFIKMLSA ETLVGMGELR

 Sec18p TCVEGGMMLY SERVNSILKN GARYVRQVRE SDKSRLVSLI IHGPAGSGKT ALAAEIALKS GPPFIRLISP NELSGMSESA

 Nsf SYIMNGIIKW GDPVTRVLDD GELLVQQTKN SDRTPLVSVL LEGPPHSGKT ALAAKIAEES NFPFIKICSP DKMIGFSETA

C_Sec18p KIQEIDNVFR DVHKSPLNVL VIDKIENIIN YNPIGPRFSN DILQVLMVYL TKKPPKGRRL LIIGTTSQYQ VFKHMLNIDS

 Sec18p KIAYIDNIFR DAYKSPLNIL VIDSLETLDV WVPIGPRFSN NILQMLKVAL KRKPPQDRRL LIMTTTSAYS VLQOMDILSC

 Nsf KCQAMKKIFD DAYKSQLSCV VVDDIERLLD YVPIGPRFSN LVLQALLVLL KKAPPQGRKL LIIGTTSRKD VLQEMEMLNA

C_Sec18p FNDIAVPPPI KHIEEVGKVL DKLGMNKSE REEILSQLSR Y..DINIGIK SLIDVIMVS. .KYSRDTVDE VVNNIVEKMS G....

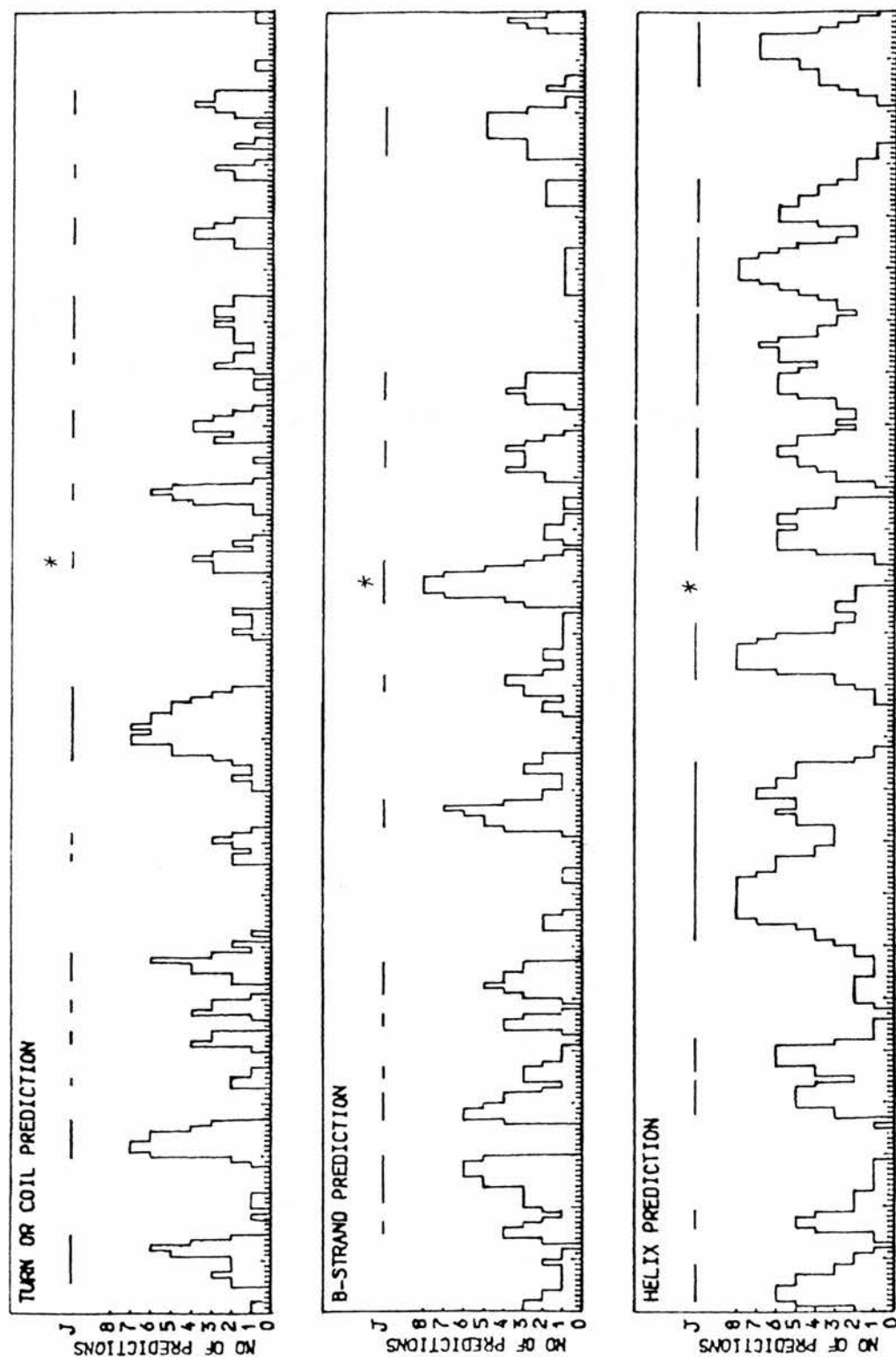
 Sec18p FDNEIAVPM TNLDELNNVM IESNFLDDAG RVKVINELSR SCPNFNVGK KTLTNIETA. .RHDEDPVNE LVELMTQSA.

 Nsf FSTTIHVPNI ATGEQLLEAL ELLGNFKDKE RTTIAQVVK. .GKKVWIGIK KLIMLIEMSL QMDPEYRVRK FLALLREEGA SPLDFD

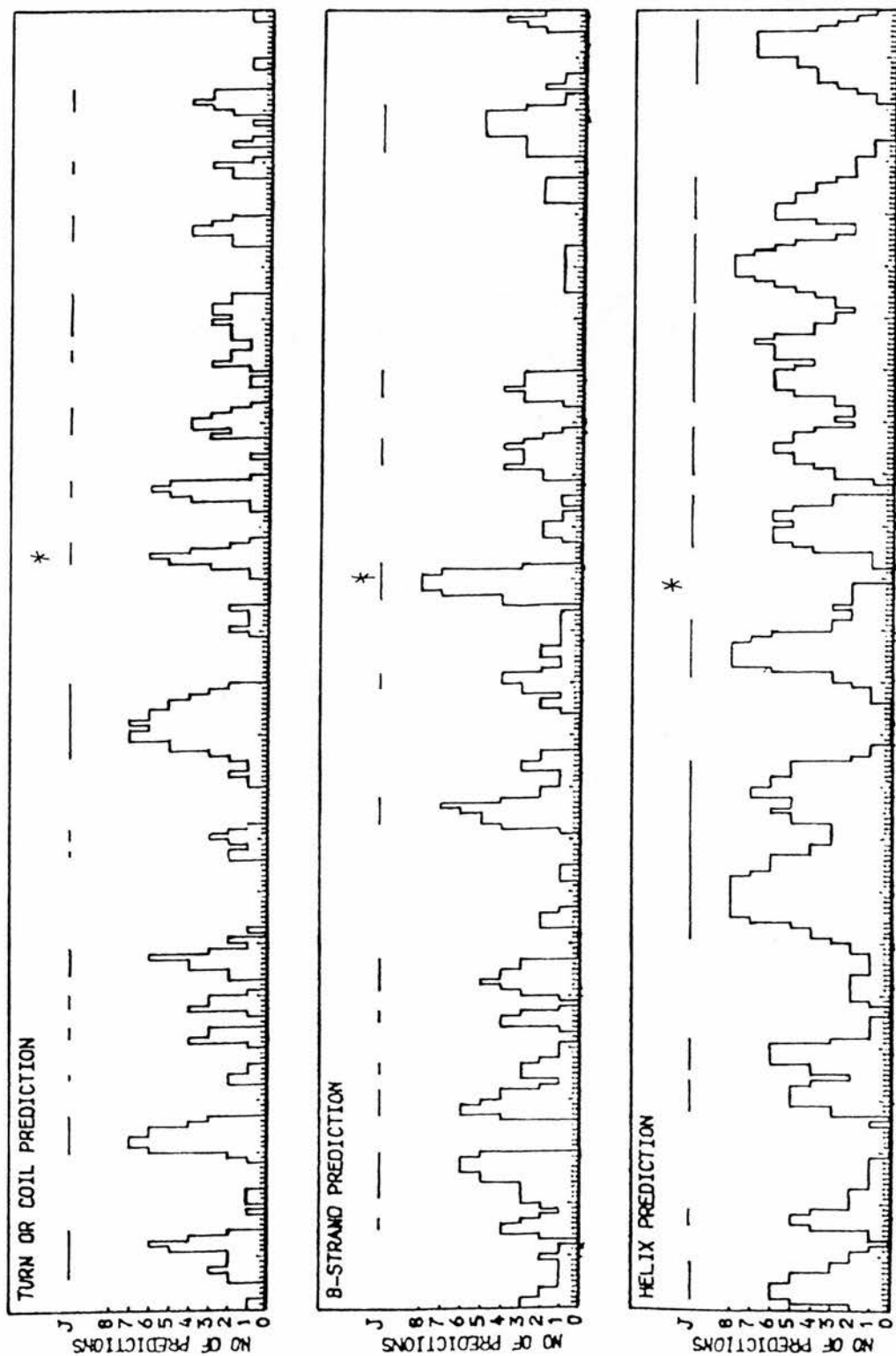
Figure 4.6 The predicted secondary structure of the protein encoded by the mutant allele SEC18-109

A secondary structure prediction of the region containing the single amino acid mutation Thr₃₉₄ to Pro was undertaken using a combination of several separate methods. Combined predictions for α -helix (bottom), β -sheet (centre) and reverse turn (top) for (a) the wild-type Sec18p and (b) the DNM mutant Sec18p are shown. The vertical axis represents the number of separate predictions for each structural element and the horizontal axis gives the residue number over a 250 amino acid stretch of the protein around the mutation of interest (see **Figure 4.3**). The horizontal lines represent the predicted structural feature from the eight separate predictions (J). * denotes the region in which the mutation occurs.

(a) Wild-type Sec18p



(b) *Class I DNM Sec18p encoded by SEC18-109 mutant allele*



Chapter 5
Characterization of the dominant negative
forms of the Sec18p

5.1 Introduction

Using a screen based upon lack of colony growth on galactose selective medium 5 DNM alleles of the *SEC18* gene have been isolated and named *SEC18-108*, *109*, *110*, *111* and *112* (see Chapter 4). To classify these mutants a number of specific approaches were employed using a range of biochemical and morphological techniques.

5.2 Biochemical Analysis

5.2.1 Growth of mutants

Growth was monitored for a wild-type yeast strain harbouring an extra copy of the *SEC18* gene under control of the *GALI* promoter, JRY188(pCH7) and for five yeast strains each harbouring a mutant copy of the *SEC18* gene under control of the *GALI* promoter, JRY188(pCH8 to pCH12). Yeast cultures were initially grown in a glycerol / ethanol based selective medium followed by an additional 20hrs in a galactose based medium which induces the expression of the DNM form of the Sec18p. As can be seen in **Figure 5.1**, the growth of the wild-type yeast strain is stimulated in the presence of galactose however growth is inhibited under similar conditions in cultures of yeast that harbour a DNM form of the *SEC18* gene. This inhibition in growth varies for each yeast strain; yeast strains JRY188(pCH8, 9, 10 and 12) show the most severe growth defect, whereas the strain JRY188(pCH11) shows a milder growth defect.

5.2.2 Secretory phenotype of the mutants

To confirm that the DNM forms of the Sec18p are affecting the secretory pathway of the yeast cells, secretion of the bacterial protein β -lactamase (expressed from the plasmid pYJS50) was monitored within the strain, JRY188(pCH7) and 5 mutant yeast strains, JRY188(pCH8 to 12), over a 12hr time course. β -lactamase has been shown to be correctly processed within yeast cells and by looking at the relative levels

of activity both internally and externally has been shown to be correctly secreted from yeast cells into the external medium (per. comm. A. Boyd). From repeated quantification of internal and external β -lactamase activities in these strains the dominant negative mutant forms of the *SEC18* gene can be classified into two main groups; Class I mutants (*SEC18-108*, *109*, *110* and *111* mutant alleles carried on the plasmids pCH8, 9, 10, 11) show a decrease in the growth rate of the yeast culture and an internal accumulation of β -lactamase when grown in galactose medium and Class II mutant (*SEC18-112* mutant allele carried on the plasmid pCH12) which shows a decrease in growth rate of the yeast culture but no internal accumulation of β -lactamase when grown in galactose medium.

The results represented in **Figure 5.2** show β -lactamase activities obtained for a typical Class I mutant (representative of the group) and a Class II mutant strain; JRY188(pCH9) and JRY188(pCH12) respectively. This data is compared to that of the wild-type strain JRY188(pCH7). The internal β -lactamase activity is represented as a percentage of the total activity present and is normalised with respect to the growth of each strain. The Class I mutant strain shows an internal accumulation of β -lactamase activity, accumulating 20% of total β -lactamase activity internally after 10 hours of growth on galactose-containing medium. This suggests that the dominant negative form of the Sec18p produced in this mutant may be interfering with the normal secretion of β -lactamase from the cell.

The phenotype exhibited by the Class II mutant is consistent with a growth defect but does not accumulate internal β -lactamase activity and so is not consistent with a classical secretory defect at this point of the analysis. From the actual data (see **Table 5.1**) it is apparent that Class I and Class II mutants have a decreased growth rate on galactose medium which results in a decrease in the external β -lactamase activity in both cases compared with that of the wild-type strain, JRY188(pCH7).

However, unlike a Class I mutant the Class II mutant does not have a corresponding increase in internal β -lactamase levels due to this imposed block on the secretory pathway. This lack of internal accumulation of β -lactamase activity is not thought to be due to incorrect processing of the prepro- α f- β -lactamase based on previous work which showed that this precursor is still active (internally and externally) in a *kex2* mutant strain (per. comm. A. Boyd). However the lack of internal activity could be due to mislocalization of β -lactamase as it is transported through the secretory pathway to an environment, such as the yeast vacuole, in which it may be degraded.

5.2.3 *Density increase of the mutants*

If one assumes that the secretory pathway is being blocked by the action of the mutant form of Sec18p within each class of mutants the subsequent internal build up of proteins and membranes should increase the density of the yeast cell. Such density enrichment experiments were used in the identification of the original *sec* mutants (Novick *et al.*, 1980) and can be used to confirm the secretory phenotype of the two classes of mutants within this study.

As can be seen in **Figure 5.3**, the control yeast strain, JRY188(pCH7), expressing wild-type Sec18p does not show a significant increase in cell density when grown in galactose medium. However yeast strains expressing Class I (expressed from plasmids pCH8 to 11) or Class II (expressed from plasmid pCH12) mutant forms of the Sec18p show an increase in density when grown in galactose medium compared to that observed when they are grown in raffinose medium. In both types of mutants the growth of the strain is blocked when grown on galactose medium resulting in an internal build up of proteins and/or membranes which leads to an increase in density of the cells. Is this density shift similar for both classes of mutants? Fractionation of the control wild-type (JRY188(pCH7)) gradient and also a Class I mutant (pCH9) and a Class II mutant (pCH12) gradient was done to address this point (**Figure 5.4**).

Compared with the wild-type fractionation pattern (**Figure 5.4(a)**) both classes of mutants show a significant increase in cell density (**Figure 5.4(b) and (c)**) when grown in galactose medium. But the density increase observed for the Class II mutant is slightly less than that observed for the Class I mutant.

5.2.4 Internal processing of carboxypeptidase Y (CPY)

Sec18p has been shown to be involved at several stages of the secretory pathway mediating vesicle docking/fusion events. At which stage of this pathway are the two classes of dominant negative mutants blocking? The processing of the soluble vacuolar glycoprotein CPY and secreted α -factor has been characterised in the *sec18-1* ts mutant strain both at the permissive and restrictive temperature by following their oligosaccharide modifications and processing in the ER, Golgi complex, and the vacuole (for CPY) as described in Chapter 1. Sec18p is required for transport (and therefore processing) of the 67 kDa p1 form of CPY from the ER and also through the yeast Golgi compartments as the 69 kDa p2 form. Sec18p apparently is not involved in delivery of CPY to the vacuole, where it undergoes final processing to the 61 kDa mCPY form (Stevens *et al.*, 1982). In the absence of polyclonal antibodies against α -factor the processing of CPY was followed in both the Class I and Class II mutants grown in galactose medium and compared with that observed in the strain JRY188(pCH7). The control strain BJ464 α was used to identify the p2 form of CPY as it is a *pep4* strain which is deficient in proteinase B and therefore cannot cleave the p2 form of CPY to its final mature form in the vacuole and so accumulates the p2 form of CPY.

Figure 5.5(a) shows the internal accumulation of the mature form of CPY over a 5hr time course in the wild-type yeast strain JRY188(pCH7) due to the continued transport and processing of newly synthesised CPY through the secretory pathway *en route* to the vacuole. As shown in **Figure 5.5(b)** the Class I mutant shows an

internal accumulation of the p1 form of CPY and a dramatic increase in the levels of the p2 form of CPY. There does not seem to be a corresponding accumulation of the mature form of CPY in this mutant. The accumulation of the p1 and p2 forms of CPY can be observed after only 5 hours of growth on galactose-containing medium. This is consistent with a model in which the dominant negative form of the Sec18p is interfering with the function of the wild-type Sec18p in mediating vesicle docking/fusion events between the ER and *cis*Golgi (hence the build up of the p1 form of CPY) or between the Golgi and vacuole (hence the build up of the p2 form of CPY). In contrast, in the Class II mutant, **Figure 5.5(c)**, mature CPY continues to accumulate, but there is only a modest accumulation of p2 CPY, and apparently no accumulation of the p1 form of CPY.

5.3 Morphological Analysis

5.3.1 Electron Microscopy: Internal membranes

Study of the *sec18-1* ts mutant at the restrictive temperature under the electron microscope reveals an internal accumulation of ER membranes and the presence of 50 nm vesicles (Novick and Schekman, 1979). The wild-type yeast strain JRY188(pCH7), Class I and Class II mutant strains, JRY188(pCH8 to 11) and JRY188(pCH12) respectively were examined by thin-section electron microscopy (EM), using the stain uranyl acetate which specifically binds to membranes within the cell (see Materials and Methods) and the strains were grown (a) in glycerol / ethanol selective medium until early exponential phase of growth at which point 3% (w/v) galactose was added and the cells were grown at 25°C over a 5hr time course with samples being taken at 0hr, 1hr, 3hr and 5hr time points after galactose addition to the medium or (b) in galactose medium at 25°C for an extended period (20hrs) to obtain the terminal phenotype of the mutants. The control mutant strain CHY01 was also analysed by EM over a similar 5hr time course of growth at the restrictive temperature of 37°C although a terminal phenotype was not done for this strain.

The electron micrographs of the wild-type yeast strain JRY188(pCH7) show the presence of large extended mitochondria within the cells due to their anaerobic growth on glycerol / ethanol medium. Both the nucleus and vacuole(s) are distinguishable within these cells as is the distribution of the ER which is closely associated with the inner surface of the plasma membrane (as shown in **Figure 5.6(a)**). Within the *ts sec18-1* mutant strain CHY01 however there appears to be an internal accumulation of extended ER tubules which line the inner surface of the plasma membrane and wind through the cytoplasm of the cell. This internal accumulation of membranes seems to become prominent after 3hr of growth at the restrictive temperature of 37°C (as shown in **Figure 5.6 (b)**).

The Class I mutant strain JRY188(pCH9) displays a normal intracellular morphology after 1 hr of growth on galactose media compared with that of the wild-type strain JRY188(pCH7). However, after 3hr of growth on galactose media this mutant accumulates extensive membranes (probably ER) that wind throughout the cell cytoplasm similar to that observed within the *ts* strain CHY01. After 5hr growth on galactose medium this mutant exhibits a highly tubular network of ER which is present at the inner surface of the plasma membrane and throughout the cytoplasm where multiple connections with the nuclear membrane are seen (see **Figure 5.6(c)**).

The Class II mutant strain JRY188(pCH12) does not seem to have any obvious membrane defects, compared to the wild-type strain JRY188(pCH7), when grown for 5 hr on galactose medium as shown in the electron micrographs in **Figure 5.6(d)** yet these cells have been shown to arrest on galactose medium (see section 5.2.1.). Does the Class II mutant have any obvious phenotype under the electron microscope when grown for a longer period in galactose medium?

Figure 5.7(a) shows the terminal phenotype of the strain JRY188(pCH7) after 20hr

growth on galactose medium. The cells contain one or two large vacuolar structures and a large nucleus as depicted in **Figure 5.7(a)**. The Class I mutant strain shows extensive accumulation of internal membranes (probably extended ER) and also a large number of small vacuolar structures as shown in **Figure 5.7(b)**. In both cases the terminal phenotype is representative of that seen in the earlier time course of growth on galactose medium. However, there is an obvious phenotype in the Class II mutant after 20hr growth on galactose medium which is not seen at an earlier growth stage (see **Figure 5.7(c)**). The Class II mutant cells are very distorted; a number of cells have not undergone septation and have multiple bud growths; many appear to have abnormally thick cell wall deposition and most cells do not contain a clear internal vacuolar structure. It is therefore apparent that the Class II mutant is defective in cell secretion or cell wall deposition but there is not a clear internal membrane abnormality as in the case of the *sec* mutants that can be seen at the electron microscopic level.

5.4 Conclusion

In this chapter I have described aspects of the phenotypes resulting from the DNM forms of Sec18p being expressed within yeast cells. Yeast cells producing a Class I mutant form of Sec18p show an arrest of growth after 10 hrs of incubation on galactose-containing medium and a corresponding increase in cell density. The *SEC18* gene has been shown to participate in vesicular trafficking events throughout the secretory and endocytotic pathways, mediating vesicle fusion events. The secretion of β -lactamase from cells expressing a Class I mutant allele was monitored to find the point at which the mutant peptide may be interfering in secretion. It was found that on arrest of growth the Class I mutant accumulated approximately 20% of the total β -lactamase activity within the cells. This suggests that secretion of β -lactamase is being retarded but not completely blocked. The processing of the vacuolar protein CPY was also monitored and shifting mutant cells to medium

containing galactose. After only 5 hours of growth on this medium there was an increase in both the p1 form (ER) and to a greater extent the p2 form (Golgi) of CPY. The processing of CPY within this mutant seems to be affected at an earlier stage of growth and to a greater extent than secretion of β -lactamase suggesting that the trafficking of CPY to the vacuole is being blocked in these mutants. Although no evidence has suggested that Sec18p is involved in vesicle trafficking to the vacuole it is thought to be involved at vesicle fusion events within the secretory and endocytotic pathways within yeast cells. The Class I DNM protein could therefore in theory interfere in membrane trafficking within yeast cells at various stages. A possible vacuolar defect may be implied within these cells from the EM analysis particularly after 20 hours of growth on galactose-containing medium. Wild-type cells all show a large vacuole-like structure whereas the Class I mutant has both a large accumulation of internal membranes and a number of small aberrant vacuolar-like structures.

The Class II mutant does interfere with the growth of the yeast cells but does not appear to cause an internal accumulation of β -lactamase activity or precursor forms of CPY. However, expression of the Class II mutant does produce an increase in the density of the yeast cells compared to the wild-type strain JRY188(pCH7). Morphological analysis has also shown that this mutant has an unusual terminal phenotype in that the cells are obviously defective in their shape and again they contain a number of small aberrant vacuolar-like structures seen under thin-section electron microscopy compared with that of the wild-type cell.

Figure 5.1 Growth of mutants upon shifting to galactose medium

Yeast strains JRY188(pCH7) and JRY188(pCH8 to pCH12) were grown overnight at 25°C in a glycerol/ethanol based medium until the O.D₆₀₀ was approximately 0.2.

The O.D₆₀₀ was monitored for a further 4hrs in this medium at which point 3% (w/v) galactose was added. The growth of the cultures was monitored for a further 20hrs.

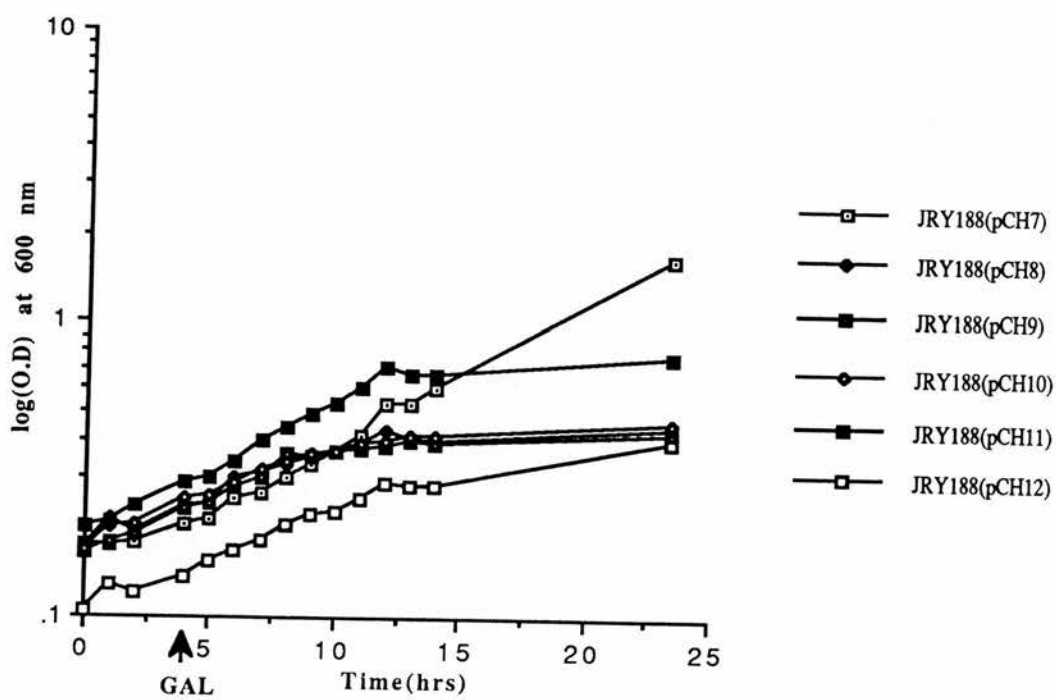


Figure 5.2. Assay of β -lactamase activity secreted from both wild-type and mutant yeast strains.

Yeast strains JRY188(pCH7) and JRY188(pCH8 to 12) were transformed with the plasmid pYJS50 (carrying a prepro- α f- β -lactamase fusion gene under the control of the α -factor promoter and the *LEU2* selective marker) which constitutively expresses the bacterial enzyme β -lactamase. These transformants were grown in 200ml cultures of 3% glycerol/ethanol selective medium overnight at 25°C until the O.D₆₀₀ was approximately 0.2 (t_0). After 2hrs of growth at 25°C, galactose was added to a final concentration of 3% (w/v) and the cultures grown for a further 10hrs. Duplicate 5ml samples were removed from each culture at t_0 , t_2 , t_4 , t_6 , t_8 , t_{10} , and t_{12} and the O.D₆₀₀ measured. Each aliquot was centrifuged at 5,000 rpm for 5 min to pellet the yeast cells and the external medium was retained. The β -lactamase activity was measured in a low speed supernatant (S3), prepared from a glass bead homogenate, containing 0.1% (v/v) Triton X100 and termed the internal β -lactamase activity. The β -lactamase activity was also measured in the external medium (see Materials and Methods). The internal β -lactamase activity was measured as a fraction of the total β -lactamase activity present. The two graphs presented are representative of results obtained for Class I and Class II mutants and are compared to that of the strain JRY188(pCH7):

GRAPH (a) : Internal accumulation of β -lactamase observed for Class I mutants compared with the strain JRY188(pCH7).

GRAPH (b): Internal β -lactamase activity observed for the Class II mutant compared with the strain JRY188(pCH7).

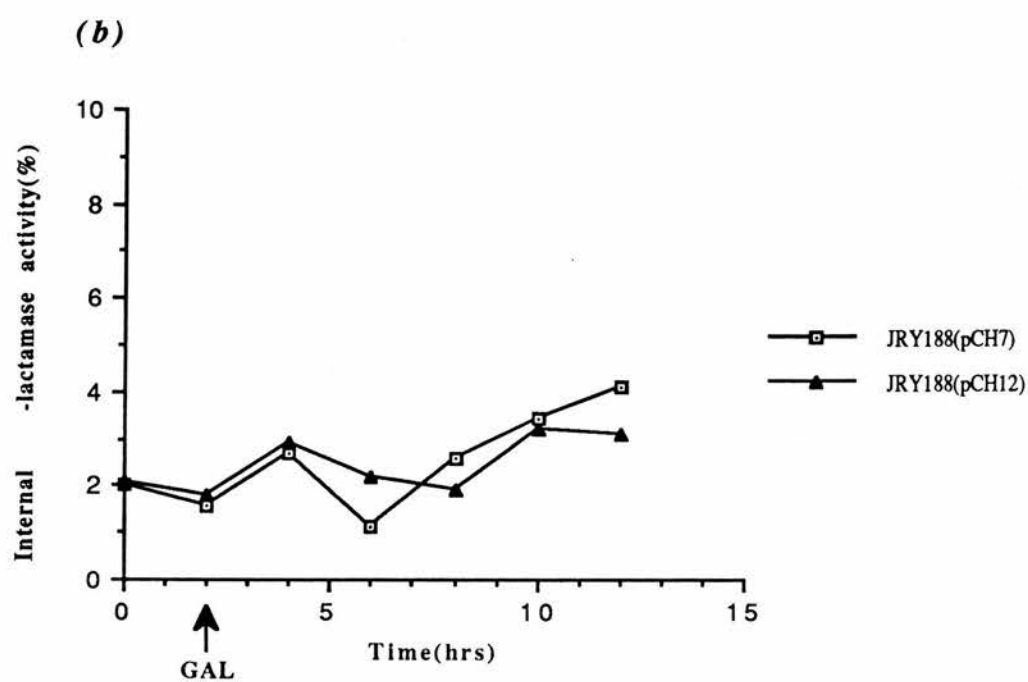
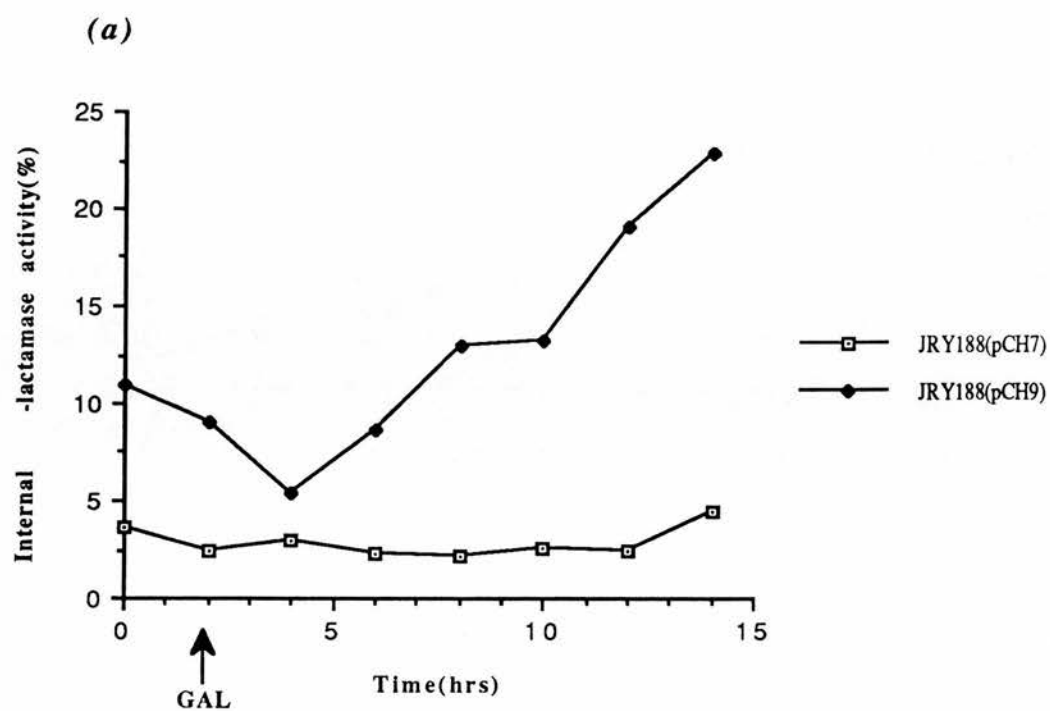


Table 5.1. Internal and external β -lactamase activity within a Class I and Class II mutant. This table presents two sets of data comparing internal and external levels of β -lactamase activity. pCH7₁ represents control data for pCH9 and pCH7₂ represents control data for pCH12 in individual experiments.

Time (hrs)	pCH7 ₁		pCH9		pCH7 ₂		pCH12	
	Int	Ext	Int	Ext	Int	Ext	Int	Ext
0	.027	.707	.119	.970	.012	.555	.012	.542
2	.021	.817	.146	1.45	.016	.989	.018	.956
4	.044	1.43	.162	2.84	.030	1.09	.039	1.31
6	.035	1.53	.227	2.39	.013	1.22	.023	1.01
8	.063	3.02	.271	1.82	.029	1.09	.018	.910
10	.056	2.12	.252	1.66	.049	1.39	.035	1.04
12	.058	2.29	.367	1.55	.064	1.48	.024	.750

KEY: Int - internal β -lactamase activity (ΔA_{490} per min per ml per OD₆₀₀)

Ext - external β -lactamase activity (ΔA_{490} per min per ml per OD₆₀₀)

pCH7 - Yeast strain JRY188(pCH7)

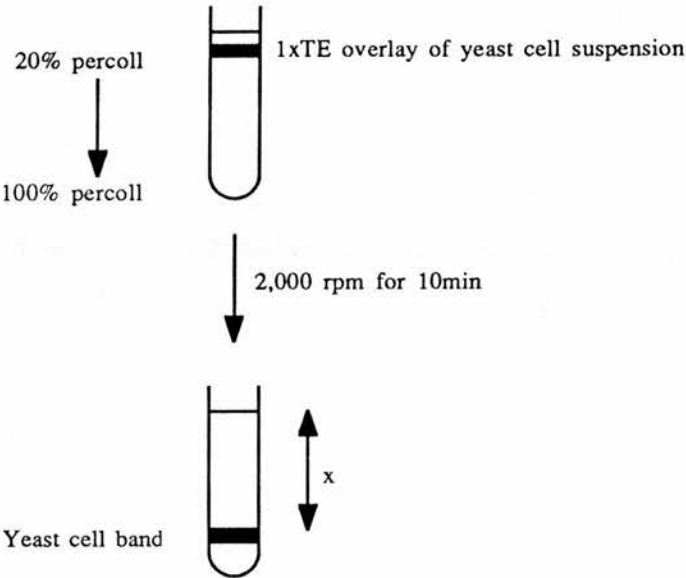
pCH9 - Yeast strain JRY188(pCH9)

pCH12 - Yeast strain JRY188(pCH12)

Figure 5.3. Density enrichment of mutant strains grown on galactose medium.

The yeast strains JRY188(pCH7) and JRY188(pCH8 to 12) were grown in 10ml of raffinose selective medium at 25°C until the OD₆₀₀ was 0.2. The cultures were split into two 5ml cultures (A and B) and galactose was added into culture B to a final concentration of 2% (w/v). These cultures were grown for a further 5hrs at 25°C at which point the cells were pelleted by centrifugation at 5,000rpm for 5 min. The cell pellets were resuspended in 500µl of 10% Percoll, 1xTE (10 mM Tris.HCl pH7.4, 1 mM EDTA pH8.0). This 'yeast cell suspension' was layered on top of a 20 - 100% Percoll/1xTE continuous gradient (10ml) then overlayed with 500µl of 1xTE buffer as shown in (*a*). The gradients were centrifuged at 2,000rpm for 10min at room temperature. The distance from the top of the gradient to the middle of each yeast band within the gradient was measured as a fraction of the gradient size and is represented in (*b*).

(a)



(b)

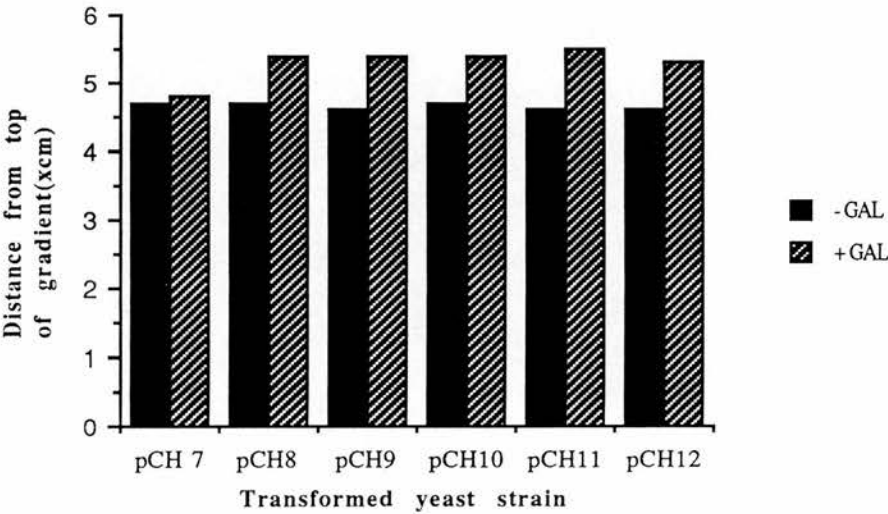


Figure 5.4. Fractionation of density gradients.

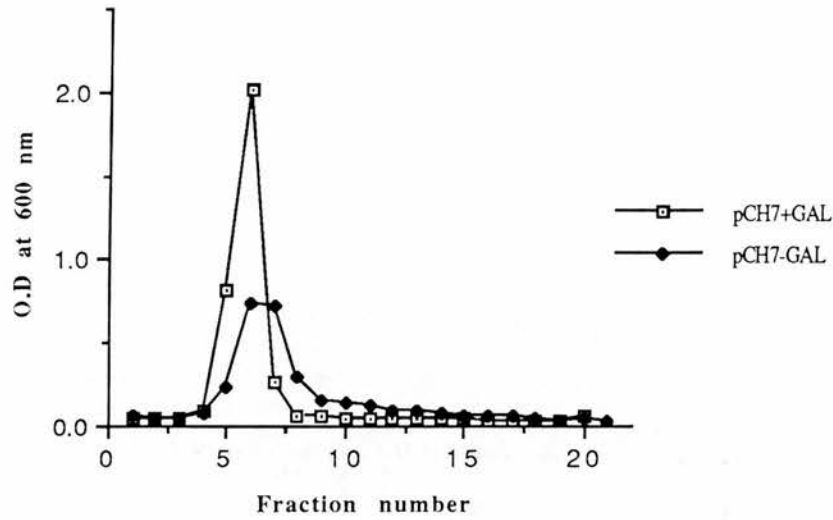
The gradients from yeast transformants JRY188(pCH7), JRY188(pCH9) and JRY188(pCH12) grown in raffinose (-GAL) or galactose (+GAL) medium were fractionated by puncturing the bottom of the tube containing the gradient and pumping twenty 0.5ml fractions from the gradient. The O.D₆₀₀ was recorded for each fraction and is shown on the following graphs:

GRAPH (a) : Control gradient showing a nonsignificant density shift of wild-type yeast JRY188(pCH7) when grown in galactose medium.

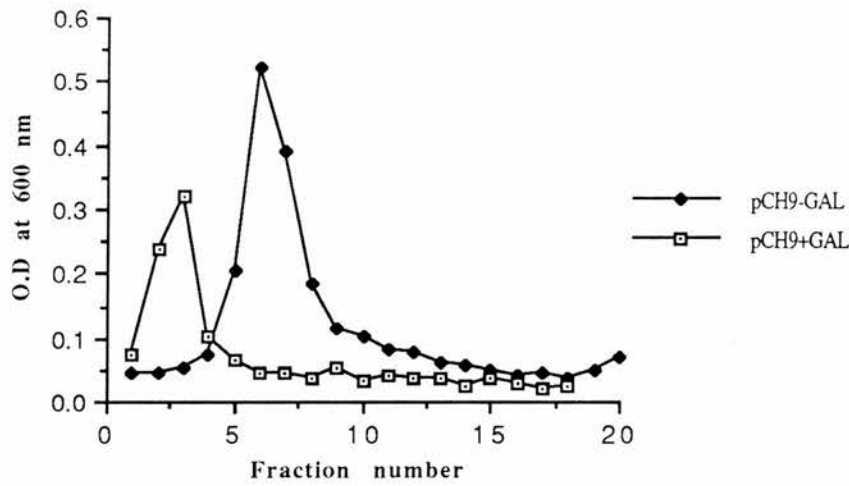
GRAPH (b) : Density shift observed for a Class I mutant -JRY188 (pCH9), in galactose medium.

GRAPH (c) : Density shift observed for a Class II mutant -JRY188(pCH12), in galactose medium.

(a)



(b)



(c)

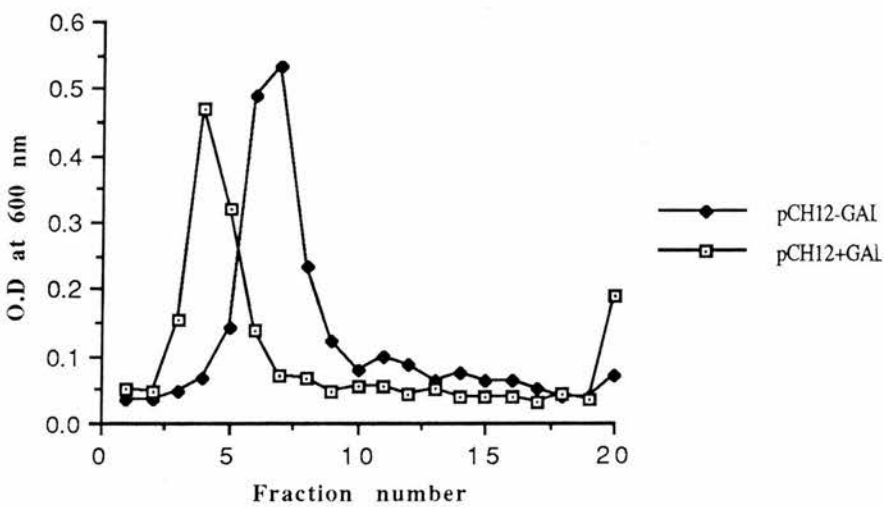


Figure 5.5. Western blot analysis of carboxypeptidase Y (CPY) processing within both wild-type and mutant yeast strains.

The yeast strains JRY188(pCH7), JRY188(pCH9) and JRY188(pCH12) were inoculated into a 150ml culture of glycerol/ethanol based selective medium which was grown overnight at 25°C until the O.D₆₀₀ was approximately 0.5 (t_0). Galactose was then added to a final concentration of 3%(w/v) and the cultures were grown for a further 5hrs. 20ml samples were removed from these cultures at t_1 , t_3 , and t_5 from which a low speed supernatant (S3) was prepared from a glass bead homogenate and resuspended in a final volume of 50 μ l. 15 μ l of each sample was loaded onto a 10% SDS polyacrylamide gel (containing a 3:1 ratio of acrylamide : bis acrylamide). CPY was detected by Western blot analysis using an affinity purified primary rabbit anti-CPY polyclonal antibody at a 1:100 dilution overnight. The blot was then incubated with a second donkey anti-rabbit HRP conjugate polyclonal antibody at a 1:5,000 dilution for 20 min and detection was done using ECL.

GEL (a) : LANE 1 to 4 contain S3 samples of strain JRY188(pCH7) at time points t_0 , t_1 , t_3 and t_5 .

GEL (b) : LANE 1 to 4 contain S3 samples of strain JRY188(pCH9) at time points t_0 , t_1 , t_3 and t_5 .

GEL (c) : LANE 1 to 4 contain S3 samples of strain JRY188(pCH12) at time points t_0 , t_1 , t_3 and t_5 .

LANE 5 in each gel contains an S3 extract from the 'terminal' time point of 20hr (t_{20}).

LANE 6 in each gel contains an S3 extract from the strain BJ464 α showing the p2 form of CPY.

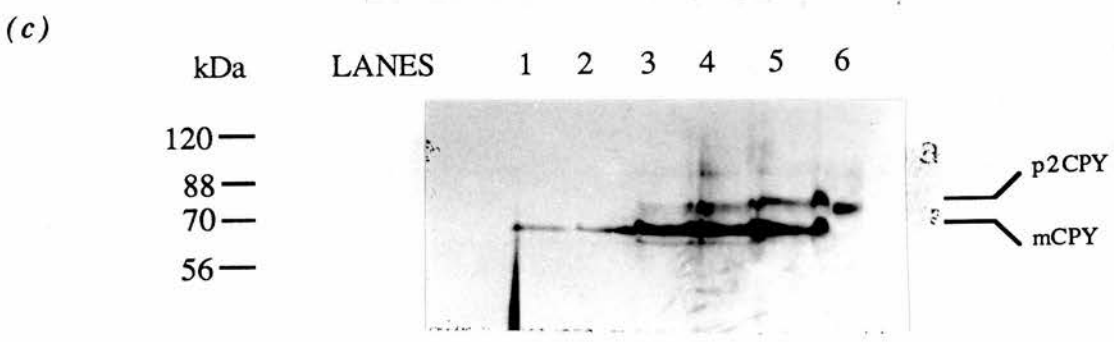
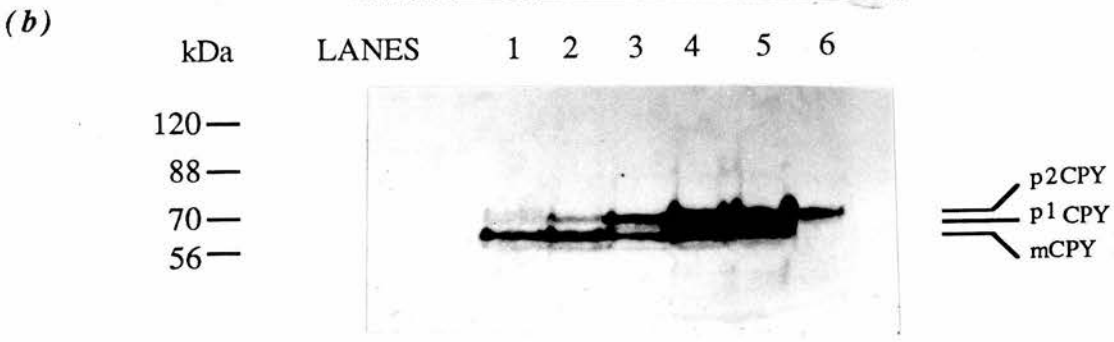
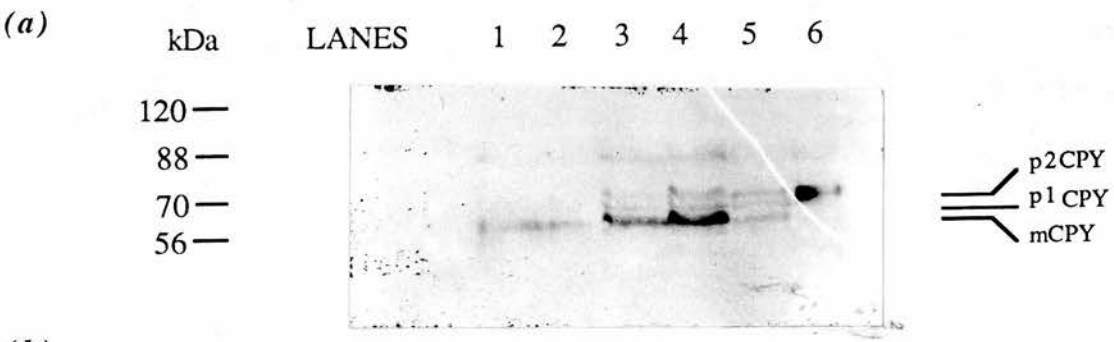


Figure 5.6. Electron micrographs of wild-type and mutant yeast strains over a 5 hour time course of growth on galactose medium or at 37°C.

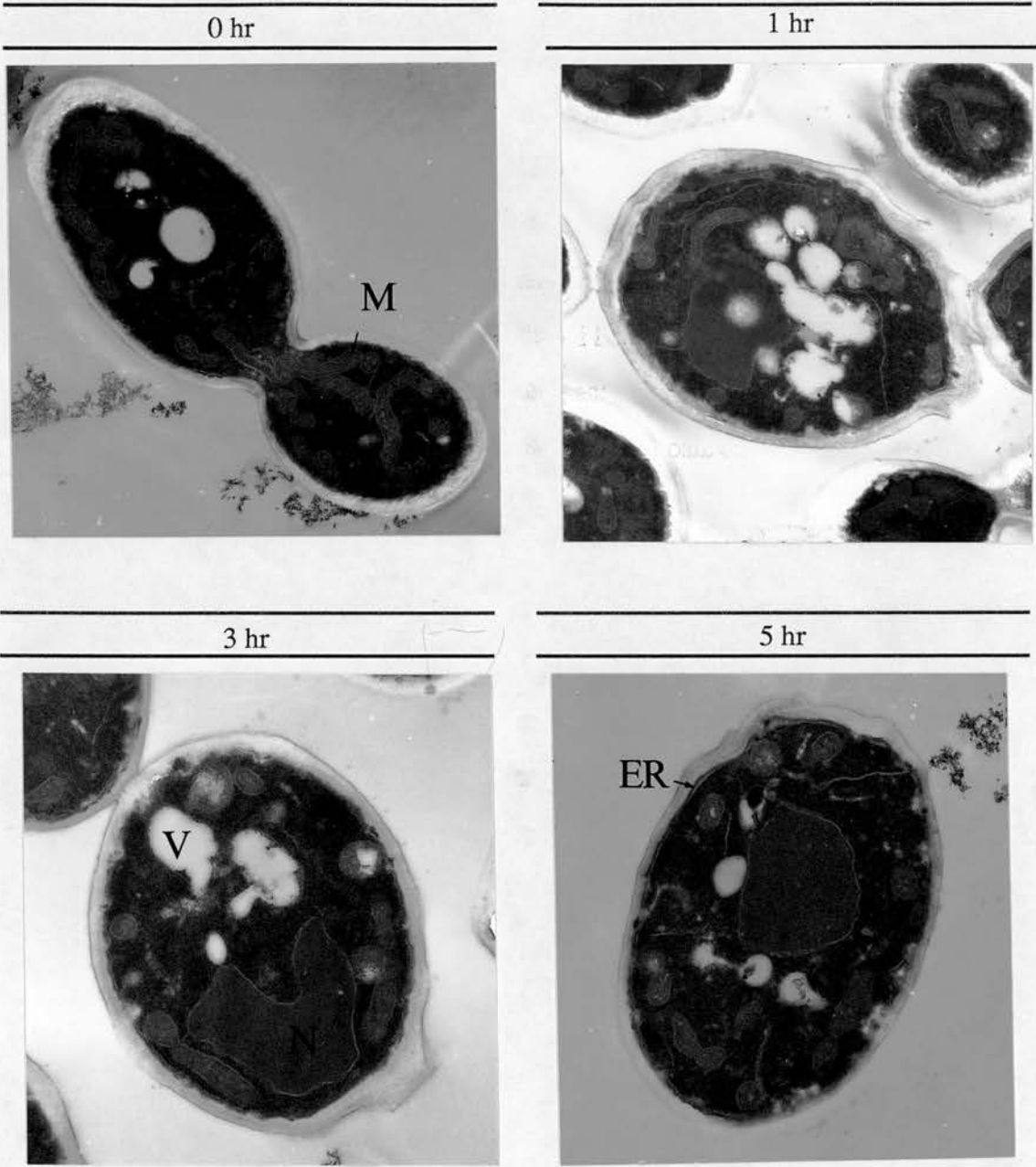
The yeast strains JRY188(pCH7), JRY188(pCH9) and JRY188(pCH12) were grown overnight in 200ml culture volumes of 3% glycerol/ethanol selective media at 25°C to a starting O.D₆₀₀ of approximately 0.2. A 50ml sample was removed (t_0) and galactose was added to a final concentration of 3%(w/v). The cultures were then grown for a further 5 hrs at 25°C with 50ml samples removed at t_1 , t_3 and t_5 . These aliquots of cells were immediately fixed in solution and the cells prepared for electron microscopy after staining in uranyl acetate as described in Materials and Methods.

The yeast strain CHY01 was also monitored over a 5hr growth period during a shift to the restrictive temperature of 37°C. The resultant electron micrographs are represented below for the four time points (t_0 , t_1 , t_3 , and t_5):

- (a) Electron micrographs of wild-type strain JRY188(pCH7).
- (b) Electron micrographs of control strain CHY01.
- (c) Electron micrographs of a Class I mutant strain JRY188(pCH9)
- (d) Electron micrographs of a Class II mutant strain JRY188(pCH12).

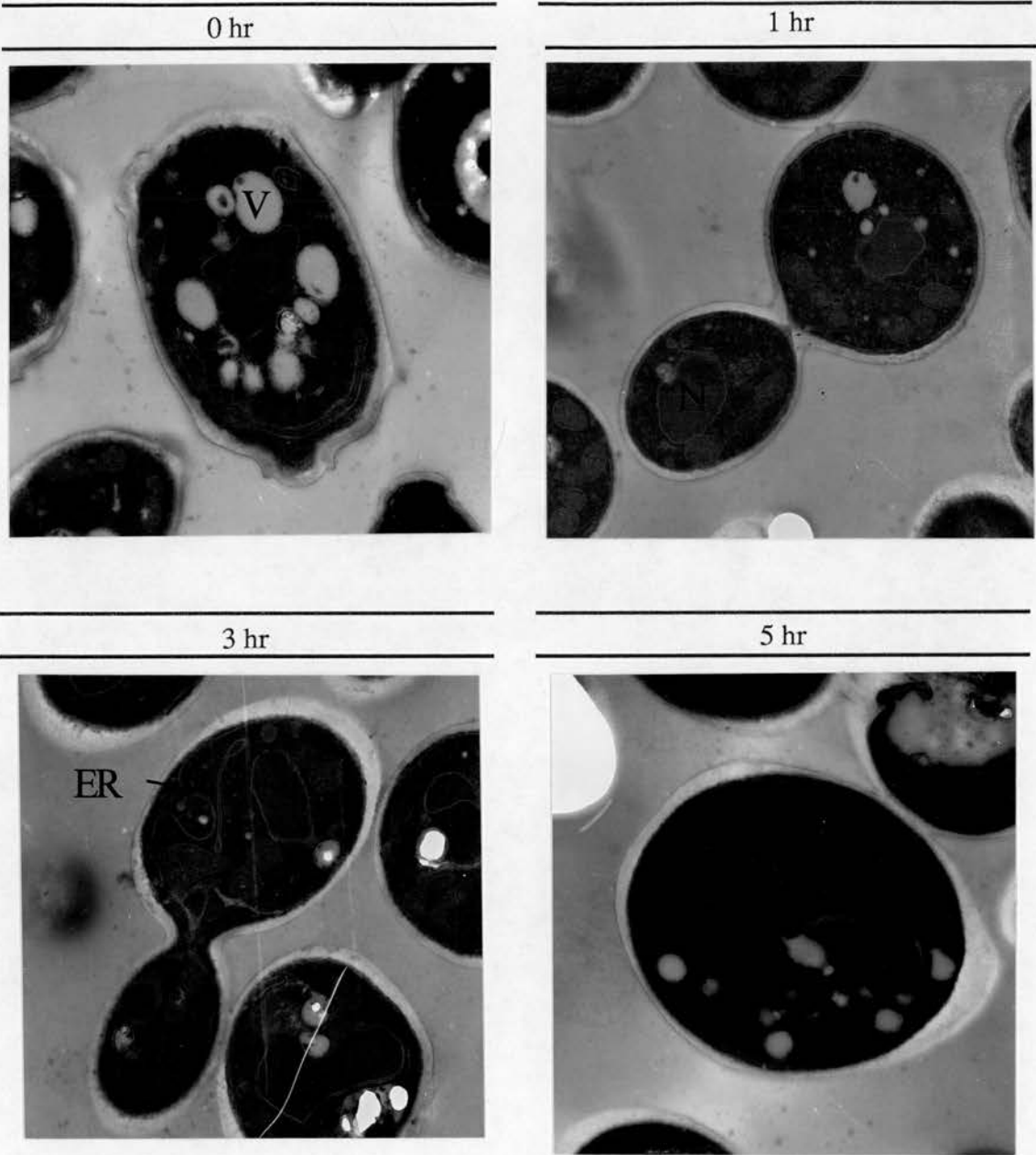
All electron micrographs were taken at a magnification between 13K and 20K.

Figure 5.6 (a)



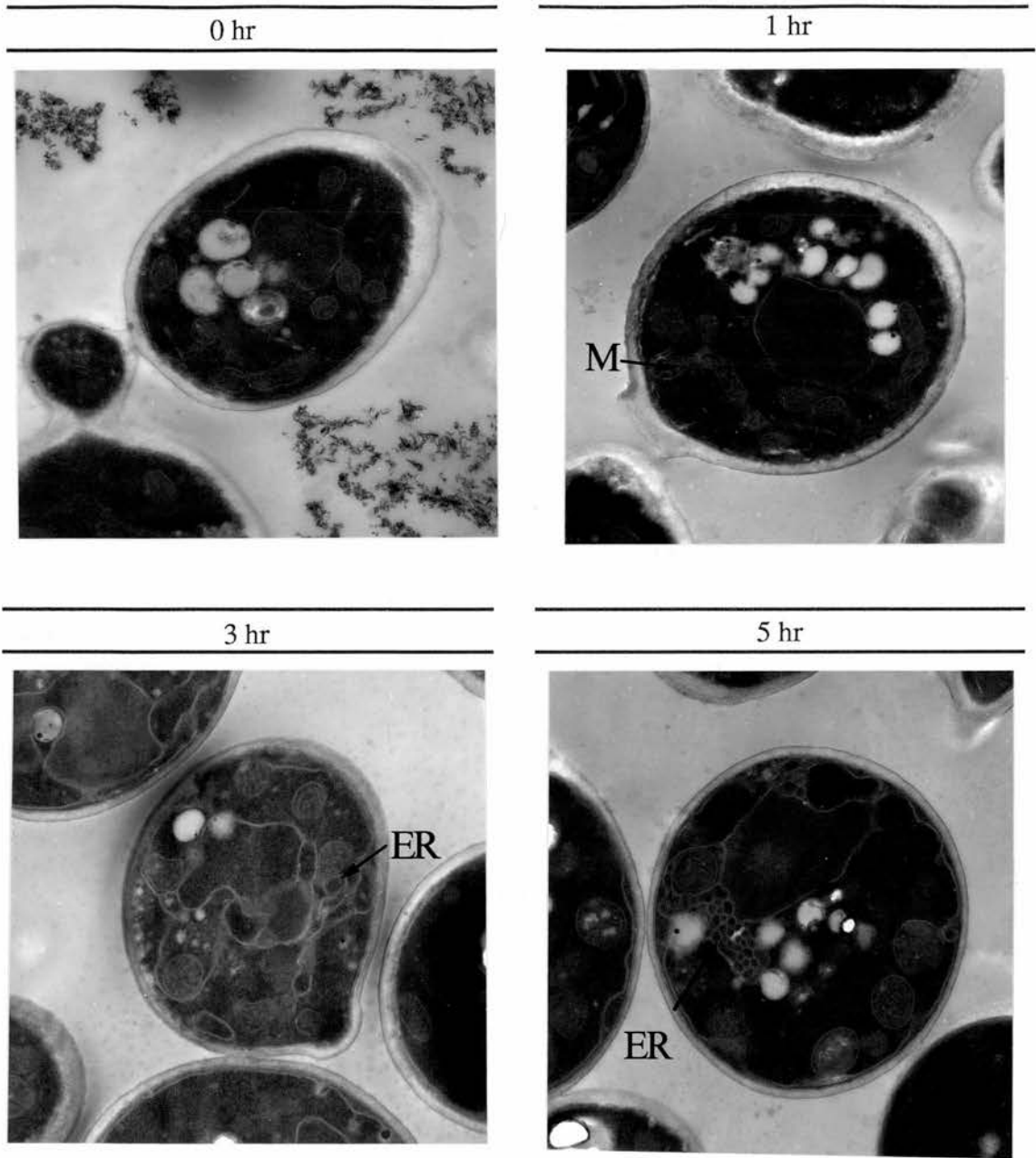
KEY: N nucleus
M mitochondrion
ER endoplasmic reticulum
V vacuole

Figure 5.6 (b)



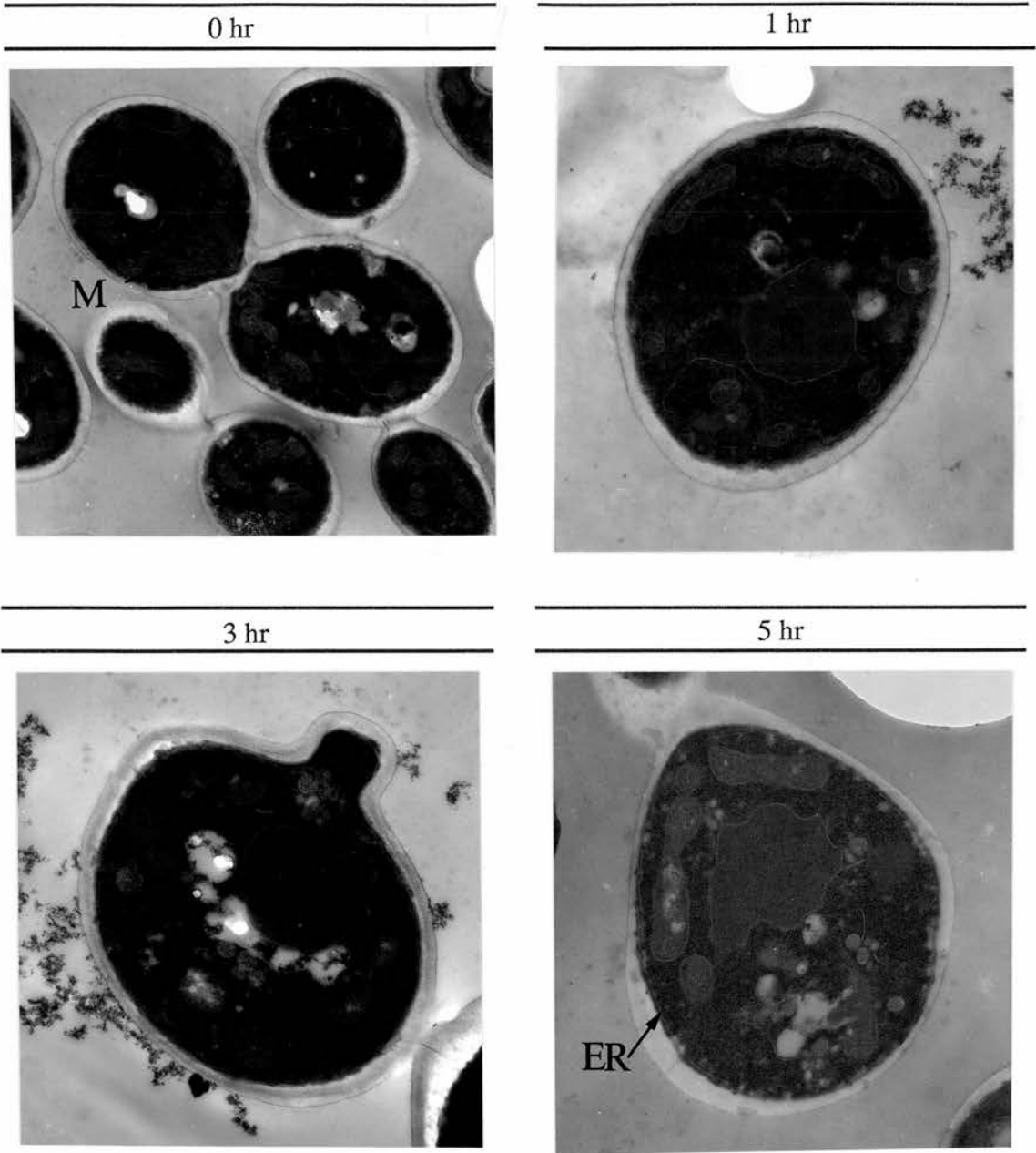
KEY: N nucleus
M mitochondrion
ER endoplasmic reticulum
V vacuole

Figure 5.6 (c)



KEY : N nucleus
M mitochondrion
ER endoplasmic reticulum
V vacuole

Figure 5.6 (d)



- KEY: N nucleus
M mitochondrion
ER endoplasmic reticulum
V vacuole

Figure 5.7 Electron micrographs of wild-type and mutant yeast strains showing their terminal phenotype after 20 hours of growth on galactose medium.

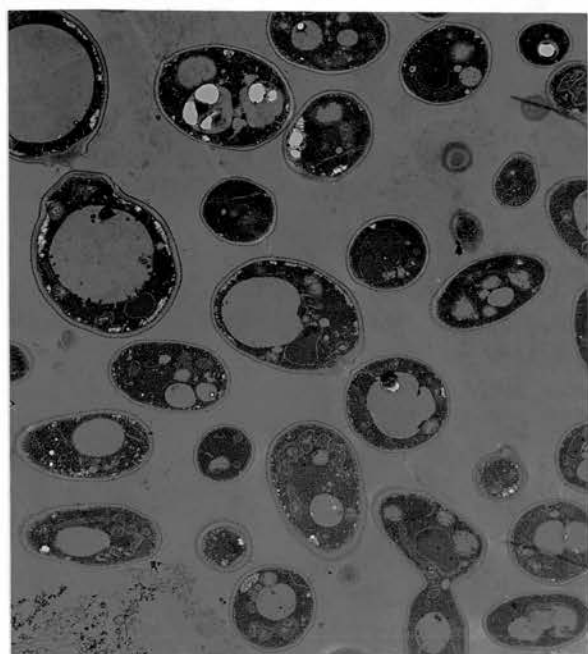
The yeast strains JRY188(pCH7) and JRY188(pCH8 to 12) were grown at 25°C overnight in 100ml 3% glycerol / ethanol selective medium to a starting O.D₆₀₀ of 0.1 to 0.2. Growth of these cultures was monitored for 4hrs at 25°C then galactose was added to a final concentration of 3%(w/v) and growth was monitored for a further 20hrs at 25°C. These yeast cultures were then immediately fixed and prepared for electron microscopy as described in Materials and Methods. The resultant micrographs are as below:

- (a) Electron micrograph of wild-type yeast strain JRY188(pCH7)
- (b) Electron micrograph of Class I mutant strain JRY188(pCH9)
- (c) Electron micrograph of Class II mutant strain JRY188(pCH12)

All electron micrographs were taken at a magnification of 5K.

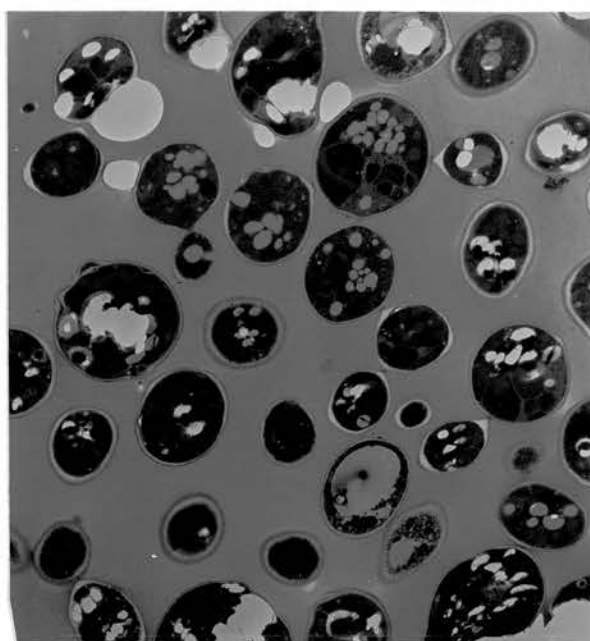
(a)

Wild-type



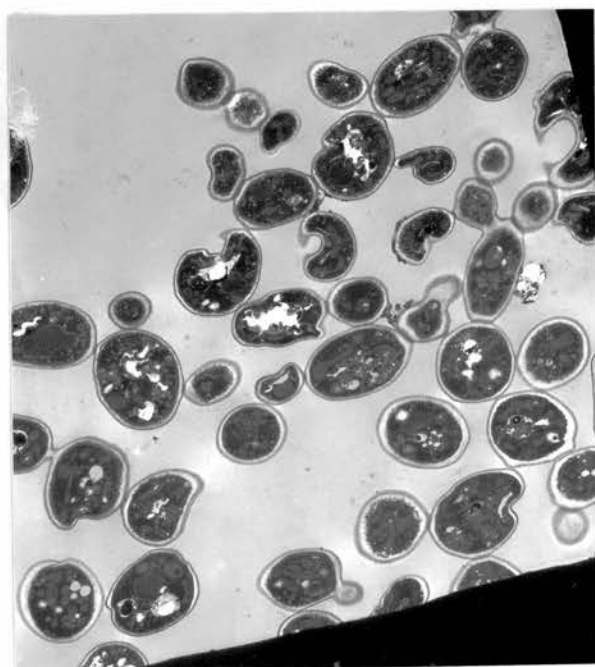
(b)

Class I



(c)

Class II



Chapter 6

***Isolation of galactose-resistant revertants of strains
harbouring dominant negative SEC18 mutations***

6.1 Introduction

The construction and isolation of dominant negative mutant forms of the *SEC18* gene has been described in Chapter 4. To try and identify genes (and ultimately proteins) which interact with the *SEC18* gene the isolation of suppressors of these mutants was undertaken. This type of genetic screen is one in which the parental mutation (the DNM form of the *SEC18* gene) and the suppressor mutation alter sites of mutual protein / protein interaction. The parental mutation destroys or distorts this protein interaction and the suppressor mutation produces a compensating alteration that restores the interaction. An intragenic suppressor produces a compensating alteration in the parental protein itself whereas an extragenic suppressor produces a compensating alteration in another protein that is in physical contact with the parental protein. Suppressor analysis is particularly useful for investigating proteins that engage in strong non-covalent interactions with one another (Moya *et al.*, 1993). Since Sec18p is thought to be involved in a protein complex the isolation of extragenic suppressors seems a good approach to identify interacting proteins. A genetic interaction has previously been shown between the *SEC18* gene and the *SEC17* gene and may be used to validate the approach taken within this genetic screen.

6.2 Isolation of suppressors of the DNM forms of the *SEC18* gene

The five dominant negative mutant forms of the Sec18p confer a secretory defect in the yeast *S. cerevisiae in vivo* which is shown as conditional lethality on galactose medium (see Chapter 4). Spontaneous suppressors or suppressors obtained by using a mutagen that overcome the original mutation can then be isolated such that the strain regains the ability to grow on galactose medium. For this study both untreated and chemically mutagenised cells (using the mutagen methanesulphonic acid ethyl ester, EMS) were used in attempts to isolate suppressors of the original mutations. Obviously, this mutagenic procedure may simply produce further

mutations in the DNM form of the *SEC18* gene which compensate for or reverse the original mutation (intragenic suppressors) and so secretion will occur as normal. To minimise this, so favouring the isolation of extragenic suppressors, yeast strains containing two copies of the DNM form of the *SEC18* gene (one copy being carried on a centromeric based plasmid and the other being integrated into the genome) (**Figure 6.1**) were used in the mutagenic procedure.

The extra copy of the DNM *SEC18* gene was integrated into the genome of the yeast strain JRY188 at the *URA3* locus. The *ura3-52* allele within this yeast strain is caused by an insertion of the transposable (Ty) element within the coding region of the *URA3* gene (Rose and Winston, 1984). Following integrative transformation these strains were shown to have a mutant phenotype of conditional lethality when grown on galactose selective medium and the disruption of the *ura3-52* allele was verified by Southern blot analysis (**Figure 6.2**). Although some integrated strains don't show all the expected fragments on Southern blot analysis, each integrated strain has a mutant phenotype of lack of growth on galactose medium. These strains were named CHY02 to CHY06 respectively.

The yeast strains CHY02 to CHY06 were then transformed with the corresponding DNM form of the *SEC18* gene carried on the plasmids pCH8 to pCH12 thus creating strains which have two copies of the DNM form of the *SEC18* gene: these strains were named CHY02(pCH8) to CHY06(pCH12) respectively. Initial studies were undertaken to isolate suppressors of two of these mutant strains CHY03(pCH9) and CHY06(pCH12) which express mutant forms of the Sec18p that have been characterized to the greatest extent (see Chapters 4 and 5) and represent a Class I and Class II mutant respectively. Both of these strains were chemically mutagenised (see Materials and Methods) and then after a recovery period were plated out onto galactose selective plates. It was hoped that any

colonies that grew on galactose would contain a suppressor mutation in a novel gene encoding an altered protein that could compensate for the initial dominant negative mutation.

To identify the gene in which this suppressor mutation lies two possible approaches were considered; (a) A secondary phenotype conferred by the suppressor mutation could be identified such as cold or temperature sensitivity. Rescue of this secondary phenotype by a wild-type yeast expression library would then identify the wild-type copy of the original suppressor allele. (b) Alternatively, in the absence of a secondary phenotype a genomic library may be constructed from the suppressor strains so identifying the suppressor allele directly. The latter approach was taken in this work.

A number of revertants of both strains were isolated (>100) and named CHY02/Sx(pCH9) and CHY06/Sx(pCH12) where x=number of suppressor. These strains could harbour either dominant or recessive suppressor mutations. Since a large number of suppressors were isolated I decided that rather than construct individual genomic libraries for each suppressor a pooled genomic library would be a more reasonable approach. This approach also favours the identification of dominant suppressor alleles. The construction of an initial genomic library however was attempted in order to optimise the techniques involved. Unfortunately problems arose in the construction of this library and so I will describe the approaches taken in constructing such a library. These attempts were, however, ultimately unsuccessful.

6.3 Large scale purification and size fractionation of yeast genomic DNA (Rose et al., 1987)

The suppressor yeast strain CHY02/S72(pCH9) was grown overnight at 30°C in a 1L culture volume of YEPD medium. The yeast culture was centrifuged at 5,000 rpm for 10min and the pellet was then washed in 500 mM EDTA pH7.5. The resultant pellet was then resuspended in 5 ml of lysis buffer (0.1 M Tris.HCl pH9.5, 0.15 M NaCl, 0.1 M EDTA, 2%(v/v) β-mercaptoethanol) and frozen in liquid nitrogen for 15-30 min. This sample was then thawed and an additional 20 ml of lysis buffer pH9.5 and 4%(w/v) sarcosyl was added and incubated at 45°C for a further 20min. To this material 20 ml of lysis buffer pH8.0 containing 4%(w/v) sarcosyl was added and incubated at 70°C for 15min. DNase-free RNase was added to a final concentration of 0.1 mg/ml and the sample incubated at 45°C for 1hr. Proteinase K was then added to a final concentration of 0.8 mg/ml and the mixture was incubated at 37°C for 2 hrs, followed by incubation at 70°C for 15min. This material was extracted twice with an equal volume of phenol/chloroform mixture (24:1) and the upper aqueous layer extracted each time after centrifugation at 10,000 rpm for 15min. This upper aqueous layer (containing the DNA) was incubated at 45°C for 2.5hr to remove any traces of chloroform then 2X volume of 100% ethanol was added to precipitate the DNA. The DNA was spooled from this mixture and resuspended in 10 ml of TE buffer. Solid CsCl was added to this DNA solution to reach a final concentration of 1g CsCl/0.8 ml. These tubes were centrifuged at 50,000 rpm for 18hr at 15°C with no brake. The tubes were perforated at the bottom and fractions collected. Fractions which contained DNA were pooled and dialysed against TE buffer for 24hr and the concentration of this DNA determined spectrophotometrically to be 0.1µg/µl then stored at -70°C.

Purified genomic DNA (1µg) from the suppressor strain CHY02/S72(pCH9) was partially digested with *Sau3A* initially using different concentrations of enzyme to

optimise the digestions to yield fragments of genomic DNA of ≥ 10 kbp. Genomic DNA was digested with 0.08U/ μ g DNA up to 2U/ μ g DNA at 37°C for 1 hour and the best concentration of enzyme was found to be 0.08U *Sau3A*/mg DNA. This was then scaled up for approximately 400 μ g of genomic DNA which was digested with 0.8U *Sau3A*/mg DNA for 1hr at 37°C. The digest was then extracted with phenol/chloroform and the DNA was ethanol precipitated and resuspended in TE buffer. This partially digested DNA was size fractionated on a preparative 10-40% sucrose gradient (Ausubel *et al.*, 1987). Fractions of 0.5ml were collected from the bottom of these gradients and analysed for the presence of DNA on a 0.4% agarose gel (**Figure 6.3**). Fractions 4-7 were pooled to yield size fragments >10 kbp and fractions 8 (8 kbp), 9 (5 kbp) were retained - these fractions were diluted, ethanol precipitated and stored at 4°C.

6.4 Attempted construction of a yeast genomic DNA library

The vector YCplac22 was chosen as a low copy number plasmid to be used in constructing the suppressor library. A centromeric based vector was chosen as it had been shown that episomal plasmids carrying wild-type *SEC18* suppressed the dominant negative phenotype seen in yeast strains CHY02 and CHY06. It was anticipated that the use of a centromeric based library would minimise the occurrence of wild-type suppression of the dominant negative phenotype. The vector YCplac22 was digested with *Bam*HI then treated with calf intestine alkaline phosphatase for 20 min at 37°C. Efficiency of phosphatase treatment was monitored by comparing the efficiency of ligation of both untreated linearized vector and dephosphorylated vector. A number of test ligations were done with varying ratios of vector : insert concentrations and ligated overnight at 16°C. The bacterial strain NM522 was initially used for transformation of these ligations using CaCl_2 prepared competent cells which have a transformation efficiency of $10^5/\mu\text{g}$ DNA when prepared by this method.

DNA was prepared from a number of transformants and digested to check the size of the insert carried (**Figure 6.4**). However, as seen in **Figure 6.4** it appears that the digests are giving smaller fragments than the initial vector whereas one would predict fragments of either the same size as the vector plus an insert fragment or a linearised clone which represents the sum of the vector and insert fragment sizes. The plasmid pCH14 which is integrated in the strain used contains an ampicillin gene and the final library selection is also based on ampicillin. Could the *Sau3A* partial digestion be digesting pCH14 and these smaller plasmids are ligating and are selected for in this protocol? To overcome this the Kan^R gene was cloned from the plasmid pCXJKan-1 into YCplac22 to give the resultant plasmid YCpKan-1 (see **Table A.4**) thus allowing a final selection purely for this plasmid. The ligations were repeated with this new vector and transformants selected on kanamycin plates. However, on restriction enzyme analysis of the subsequent clones these abnormal digest-patterns were still observed. Coupled to these inexplicable clones that were being produced the transformation efficiency with the strain NM522 was quite poor. Attempts were made to improve this efficiency both by using different protocols in the preparation of the competent bacteria and using commercially made 'library efficient' JM109 competent bacteria, however in both these cases the efficiency was quite poor.

6.5 Conclusion

I have described in this Chapter the steps taken to isolate a number of suppressors of the strains CHY03 (pCH9) and CHY06 (pCH12) to identify genes that interact with the *SEC18* gene. >100 suppressors of both these strains were isolated using the chemical mutagen EMS. These suppressors may represent mutations in protein(s) involved in a Sec18p complex, which may compensate for the dominant negative form of the peptide. Mutation of the DNM Sec18p to a non-interacting form of the protein may also suppress the mutant phenotype, although the increased

copies of the DNM gene within these strains should decrease this likelihood. Reversion of the DNM Sec18p to the wild-type peptide may also occur within these strains and in theory the more copies of the DNM gene present the greater the chance for this to occur. However in practice the probability of mutating a single specific nucleotide so reverting the *SEC18-109* allele to the wild-type gene is very low. The DNM effect of Sec18p depends on the levels of mutant peptide present within the cell compared to that of the wild-type protein. Expression of both the *SEC18-109* and *SEC18-112* genes is from the *GAL1* promoter which is dependent on activation by the *GAL4* transcription factor. Therefore any mutations in the *GAL4* gene will affect the levels of GAL induction and so the amount of DNM protein produced within the yeast cells. Such strains which have a mutated *GAL4* gene or *GAL1* promoter may be identified by poor growth on galactose-containing medium. However, within the time available for this work the gene(s) responsible for these suppressors could not be isolated. This was mainly due to technical problems arising in the construction of suppressor genomic libraries (as indicated in this Chapter).

The fact that suppressors of my DNM *SEC18* genes can be isolated indicates that these mutants may be used with a number of alternative screens to isolate suppressor genes. One such screen is based on an *ADE2/ADE3* sectoring assay (Koshland *et al.*, 1985) and has recently been used to isolate the *TIP1* gene which encodes a protein that interacts with Sec20p (Sweet and Pelham, 1993). Alternatively, the isolation of multi-copy suppressors of the dominant negative gene may be investigated. Multicopy suppression has been observed with some frequency during attempts to clone genes by complementation, and in many cases the genes identified in this way encode proteins that serve a related function or interact with the original mutated gene product (Nakano and Muramatsu, 1989; Burton *et al.*, 1993).

Figure 6.1 Integration of the DNM forms of the SEC18 gene into the yeast genome by homologous recombination

The integrative plasmids YIplac211, pCH13, 14, 15, 16 and 17 were introduced into the yeast strain JRY188 and were subsequently integrated at the *ura3-52* allele *in vivo* by homologous recombination. To stimulate recombination of these plasmids at the *URA3* locus they were digested with the restriction endonuclease *StuI* which linearizes the plasmid in the *URA3* gene. This results in the integration of plasmids pCH13 to pCH17 between a recreated functional *URA3* gene and a nonfunctional *ura3-52* allele in the genome. Resultant transformants are therefore Ura⁺ and named CHY02 to CHY06. The restriction sites shown are :- *HindIII* (H) and *StuI* (S).

Digestion of wild-type yeast (JRY188) genomic DNA with *HindIII* and hybridization of an enzymatically labelled *URA3* probe to this digested DNA should produce two fragments on Southern analysis of predicted sizes: (4.15+x)kbp and (1+y)kbp. Digestion of genomic DNA from *SEC18-108*, *109*, *110*, *111* and *112* integrated strains however will produce three fragments of the predicted sizes: (4.15+x)kbp; 3.8kbp and (7.57+y)kbp.

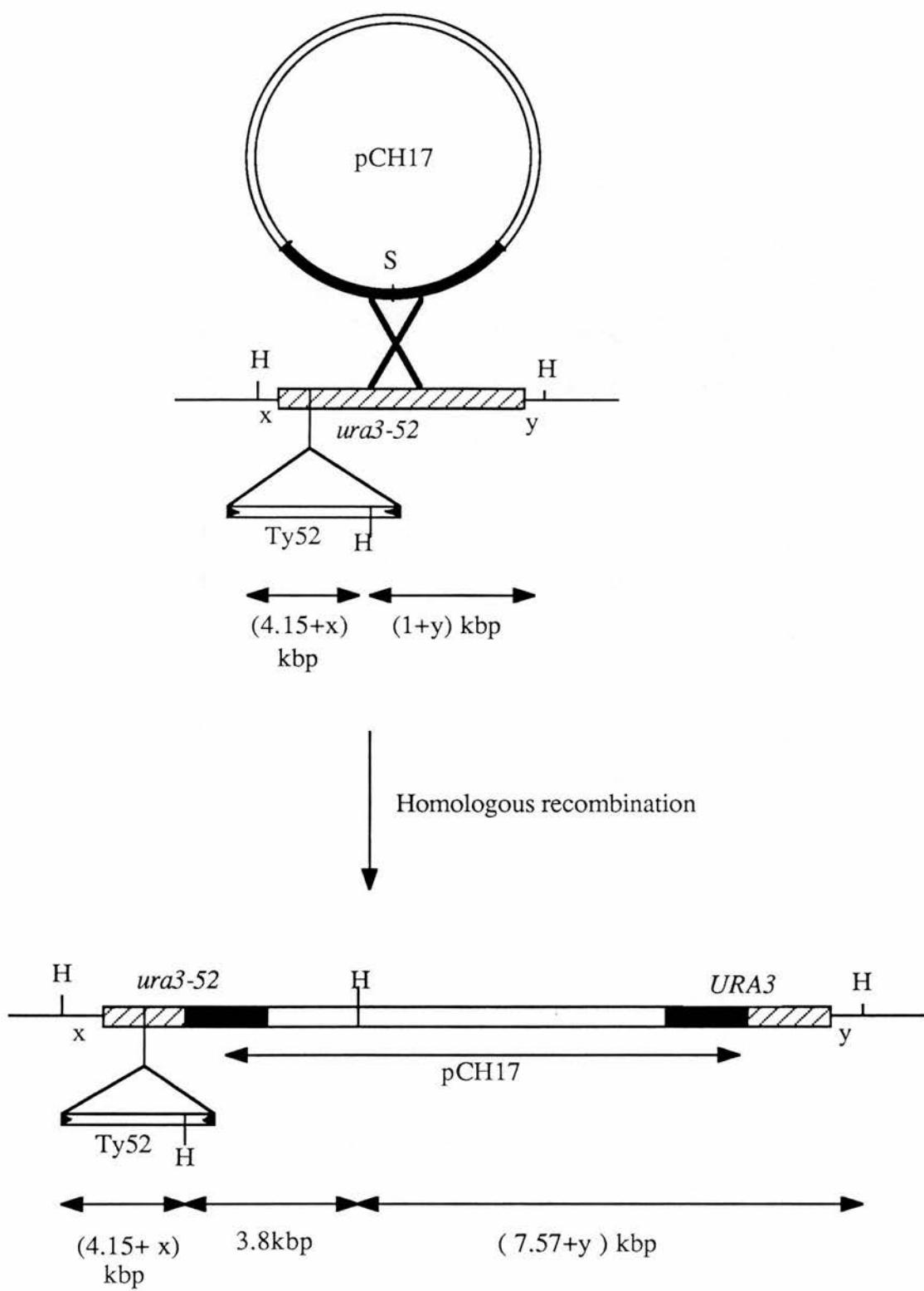


Figure 6.2 Southern blot analysis of integrated strains

The yeast strains JRY188, CHY02 to CHY06 and CHY07 were grown overnight at 30°C in YEPD medium. A small scale preparation of total genomic DNA was made from these strains and the control strain JRY188. This genomic DNA was digested with *Hind*III and electrophoresed on a 0.8% agarose gel. The DNA was transferred onto a nitrocellulose membrane and Southern analysis was performed using a nonradiolabeled DNA probe. A representative blot is shown:

LANE 1: Genomic DNA from strain CHY02

LANE 2: Genomic DNA from strain CHY03

LANE 3: Genomic DNA from strain CHY04

LANE 4: Markers-pBR 328 DNA digested separately with *Bam*HI, *Bgl*II and *Hin*FI
and mixed with each other in a ratio 2:3:3.

LANE 5: Genomic DNA from strain CHY05

LANE 6: Genomic DNA from strain CHY06

LANE 7: Genomic DNA from control strain JRY188

LANE 8: Genomic DNA from control strain CHY07

The probe used to detect the predicted DNA fragments from each strain was a 1.1 kbp *URA3* fragment obtained as a *Bam*HI cassette from the plasmid YDpU. This probe was labelled with the non-radioactive label digoxigenin-11-UTP (as described in Materials and Methods). This probe will hybridise to the three fragments of sizes; (4.15+x)kbp, 3.8kbp and (7.57+y)kbp in the *SEC18* integrated strains and to one main band of (1+y)kbp in the control strain JRY188. The marker DNA was detected by a separate probe of digoxigenin-labelled linearized pBR328 DNA.

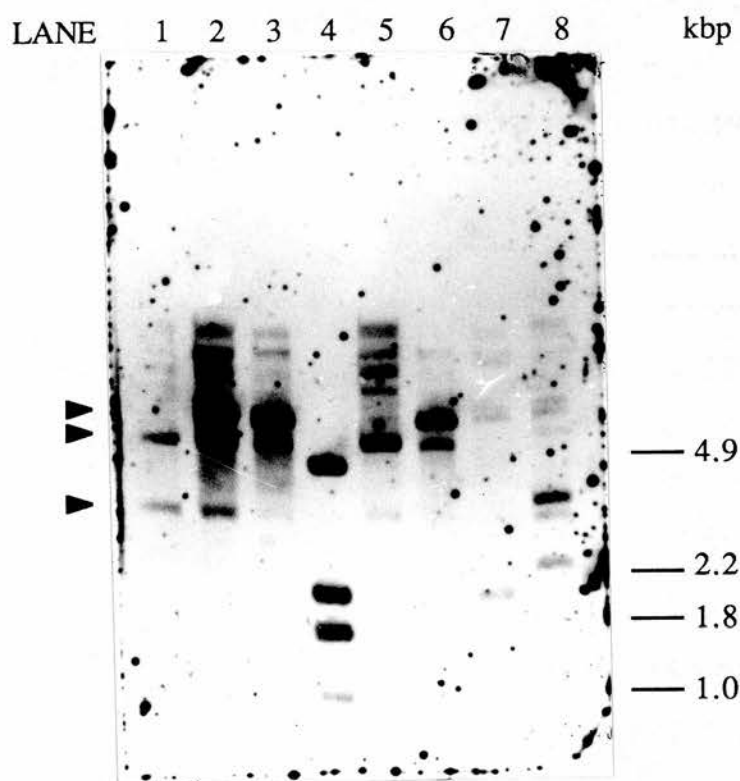


Figure 6.3 Size fractionation of partially digested yeast genomic DNA

Partially digested yeast genomic DNA was fractionated on a 10-40% sucrose gradient (as described in Materials and Methods) then the gradient was fractionated from the bottom into approximately 20 fractions. 5 μ l of each 200 μ l fraction was run on a 0.4% agarose gel and DNA was monitored in each fraction.

LANE 1 to 14: Fractions from the sucrose gradient from the bottom (lane 1) to the top of the gradient (lane 14).

LANE 16: 1kbp DNA ladder

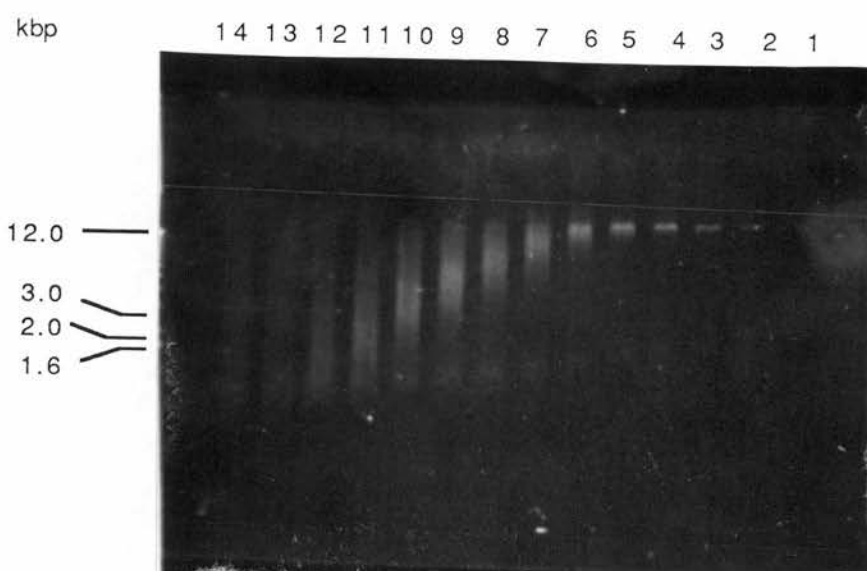
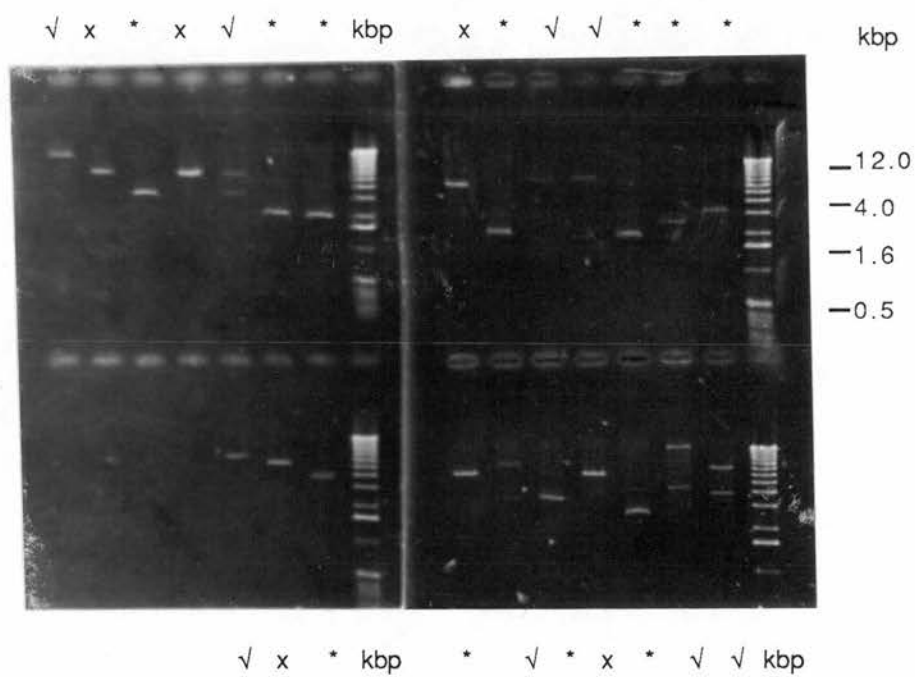


Figure 6.4 Restriction digest analysis of putative library clones

Putative genomic library clones were rescued from the transformed bacteria for restriction enzyme digestion to find the average insert size obtained. 24 clones were digested with *Eco*RI and analysed on a 0.8% agarose gel as shown. 11 clones had abnormal restriction digest patterns (*) and 6 clones did not contain a detectable insert (x). 8 recombinant plasmids contained inserts but these were only approximately 3 kbp in size (√).



Chapter 7
Discussion

7.1 Discussion

Intracellular membrane traffic is regulated by a number of mechanisms in order to facilitate the correct and efficient transport of proteins from their site of synthesis to their final destination. One of the intriguing questions posed over the last few years has been how do membrane bilayers fuse with each other and what regulates these fusion events in order to obtain targeting specificity. A combination of yeast genetics, mammalian cell free systems and affinity purification techniques has been used to answer some of these questions. Greatest progress has been achieved within mammalian systems initially using *in vitro* assays which reconstituted vesicular transport within the Golgi (see Chapter 1) apparatus. This system led to the identification of soluble proteins which were required for fusion to occur (NSF and SNAPs) which were further identified to be part of a complex of proteins which had a sedimentation coefficient of a 20S particle. Subsequent purification of this 20S particle from bovine brain material led to the identification of the individual components of this fusogenic particle involved in the regulated release at the synaptic cleft (see Chapter 1) and the finding that homologues of these proteins may act in a similar manner within a number of constitutive fusion events conserved from yeast to neuronal cells.

Within this work I have described the use of two approaches in the identification of proteins which interact with the Sec18p (NSF homologue) in the budding yeast *S. cerevisiae*. The first approach was a biochemical affinity purification procedure, relying on a tagged version of the Sec18p. A fusion protein was constructed between Sec18p and a portion of the bacterial protein, Protein A. This fusion protein was shown to be active *in vivo* by its ability to complement a *sec18-1* ts mutation at the restrictive temperature and a significant proportion of this hybrid protein fractionated in a similar fashion to that reported for the wild-type protein *in vivo*. Isolation of this Sec18pra fusion protein from yeast was achieved using the affinity of Protein A for

IgG which is bound to a Sepharose column. However, isolation of proteins complexed to this hybrid protein was unsuccessful. Firstly, the high background of non-specific proteins from yeast extract which bound to the Pra tag of the fusion protein was sufficient to mask the presence of any Sec18p-specific proteins within the affinity purified material. Secondly, although Sec18p could be identified within affinity purified material, using a polyclonal antibody raised against Sec18p, the presence of Sec17p could not be shown (again using a Sec17p specific polyclonal antibody). The lack of Sec17p within this material could be due to the small amounts of fusogenic complex being isolated in the purification procedure and therefore outwith the levels of detection employed within the assay. The presence of Sec18p within the affinity purified material is encouraging as it shows that the fusion protein is interacting with the wild-type protein. This interaction is not interfering with the normal function of Sec18p within the fusogenic complex and its capacity to facilitate vesicular transport in the secretory pathway. Therefore, I believe that an alternative affinity purification procedure is still feasible from a yeast system but an alternative tag should be used which does not show such non-specific binding of yeast proteins such as the *c-myc* epitope (Sollner *et al.*, 1993a) or dihydrofolate reductase (DHFR) tag (Salama *et al.*, 1993).

An alternative approach to that of trying to isolate complexes which have formed *in vivo* is the use of an affinity column to isolate complexes *in vitro* as has recently been reported for the affinity isolation of NSF complexing proteins from mammalian cells. This approach relied on the production of bacterially-expressed fusion proteins (NSF and α -SNAP) which were used to produce an affinity column for the purification of complexing proteins from a membrane preparation from bovine brain (Sollner *et al.*, 1993a). The alternative approach to identify proteins that complex with Sec18p would be to express a bacterial Sec18 fusion protein which could then be used on an affinity column to isolate complexing proteins. My initial studies have shown that a bacterially

expressed Protein A tag still shows this non-specific binding of yeast proteins when used in such an affinity column and so alternative tags should be looked at such as GST fusions or His₆ fusion constructs.

The second approach taken during the course of this work was a genetic approach to try and identify genes which interact with *SEC18* (apart from the *SEC17* gene). The Sec18p has been shown to contain two ATP binding sites but no other information has been reported about the nature of the interactions of this tetrameric protein within the proposed fusogenic complex. It was due to this lack of information that a random mutagenic strategy was taken in order to create Dominant Negative Mutants of the Sec18p. Mutagenesis was done using either an *in vitro* mutagenic PCR strategy or *in vivo* using a bacterial *mutD* strain. Five DNM *SEC18* genes were produced in this way which were grouped into two classes of mutations according to a number of biological and morphological criteria (see Chapter 5).

Class I mutants seem to interfere with secretion from yeast cells expressing the mutant allele (*SEC18-109* to *-III*). The mutant peptide seems to have an effect in slowing down ER to Golgi transport which was shown by an internal accumulation of the p1 form of the vacuolar protein CPY and the appearance of ER-like membranes throughout the cells. However, it was also noted that in cells expressing the *SEC18-109* mutant allele there was a large increase in the p2 form of CPY and the cells had abnormal vacuolar-like structures when viewed by EM. This suggests that vacuolar trafficking was also being affected within these cells. Analysis of CPY trafficking by pulse chase labelling may give a more precise view of CPY transport within this mutant.

The Class II mutant however does not have a build-up of secreted protein and only shows a mutant morphology under the EM after a long period of expression *in vivo*.

Obviously both classes of mutants have a dominant effect *in vivo* over the wild-type Sec18p in that the normal function of the Sec18p is impaired and secretion is blocked. This impairment of function *in vivo* may be due to the mutant protein interacting with the wild-type protein to form mixed tetramers which interact with other proteins in the fusogenic complex to give non-functional complexes or the mutant protein may just 'mop up' the wild-type protein to form non-functional aggregates which cannot interact further within a protein complex.

However what of the Class II mutant phenotype? The phenotype of the Class II mutant does not seem to be consistent with a block imposed within the secretory pathway (i.e. no internal build-up of secreted proteins, internal membranes or p1 form of CPY) yet cell growth is affected. One possibility is that the mutant form of Sec18p may be interfering in the normal proof-reading mechanism of the fusion process thus promoting non-specific fusions to occur and the mislocalization of proteins within the secretory pathway. This may be shown by the absence of accumulated internal β -lactamase activity but the decrease in external activity suggesting that maybe β -lactamase is being degraded at some point within the pathway due to its mislocalization or mislocalization of proteases to a compartment in which it resides.

To measure the types of mutations gained by the two methods of mutagenesis a Class I mutant resulting from the *mutD* strategy and the Class II mutant which underwent PCR mutagenesis were sequenced and compared to that of the wild-type gene. On resequencing of the wild-type *SEC18* gene an extra codon was identified within the sequence which has since been reported by another group and entered into EMBL data library (Van der Aart., 1994). A higher percentage of mutations were incurred when using the PCR based strategy of mutagenesis (0.5%) compared with that of the *mutD* mutagenesis (0.04%). Surprisingly in both mutants no mutations were seen within the proposed ATP binding sites. The Class I mutant has only one base change which

results in the mutation of a highly conserved threonine residue to a proline, which results in the loss of Sec18p function. The structural significance of this change is not known although initial studies show no significant alteration to the secondary structure of the protein (see Chapter 4).

A number of revertants, presumably containing suppressors of these DNMs were created. Attempts to identify the genes responsible for this suppression were unsuccessful due to a number of technical problems in the construction of genomic libraries of the suppressors. In the absence of obtaining genomic libraries further characterization of the suppressors to identify whether they are dominant or recessive alleles could be undertaken. Each suppressor strain may be crossed to a standard wild-type yeast strain (of opposite mating type). If the resultant diploid still suppressed the DNM phenotype (i.e if the strain were *GAL*⁺) then the suppressor gene would be dominant. Recessive alleles may be further sorted into complementation groups. Ideally for this two sets of suppressors should be produced, each in a strain of opposite mating type. Crossing two suppressors that lead to a *GAL*⁺ diploid suggests that the initial mutation were in the same gene. If the resulting diploid is galactose sensitive then the mutations are in different genes. In this way an idea of how many individual recessive genes (complementation groups) are present may be given.

Alternative strategies could be employed in future work to identify these suppressor genes: (1) one could look for high copy suppressors of the DNM phenotype by transforming the DNM strain with a high copy number yeast genomic library. (2) A secondary suppressor phenotype such as cold or temperature sensitivity which was linked to the suppressor phenotype could be isolated and this rescued again by a wild-type yeast genomic library.

Although proteins which interact with the Sec18p could not be identified within this work the isolation of DNM forms of the *SEC18* gene should allow the use of alternative genetic strategies in the future to identify interacting suppressor genes. The sequencing of these mutants has started to address the functional relevance of regions within the protein which are important for the function of the Sec18p *in vivo*.

Appendix

Table A.1 Bacterial strains used in this study

Bacterial (<i>Escherichia coli</i>)	Genotype	Source
NM522	<i>supE thi Δ(lac-proAB)</i> <i>hsd5 F'[proAB⁺lacI^q</i> <i>lacZΔM15]</i>	Gough and Murray (1983)
pop2136	<i>F⁻supE44 hsd R17 mcrA⁺</i> <i>mcrB⁺ r_k⁻ m_k⁺ endA1 thi-1</i> <i>aroB mal-1</i>	Kusters et al. (1989)
DH5α	<i>supE44 ΔlacU169 (Φ80</i> <i>lacZΔM15) hsdR17 recA1</i> <i>endA1 gyrA96 thi-1 relA1</i>	Gibco, BRL
BW313	<i>dut⁻ ung⁻ F'</i>	M. White

KEY:

end A1 mutation: improves plasmid yield, especially from mini-preps.

Δ(lac-proAB) deletion: removes the *lac* operon and surrounding region including two genes involved in proline biosynthesis. Most strains carry these deleted genes on an F' episome.

lacI^q mutation: results in overproduction of the lac repressor, minimizing the low level of lac expression that occurs in uninduced wild-type cells.

lacZΔM15 deletion: removes the amino-terminal α-peptide of β-galactosidase. Vectors that carry *lac* selection have a gene that encodes this peptide and rescues the mutation by α-complementation.

recA strain: is recombinant deficient and has a reduced ability to rearrange cloned sequences.

Table A.2 Yeast strains used in this study

Yeast (<i>Saccharomyces cerevisiae</i>)	Genotype	Source
JRY188	<i>MATα; leu2-3,112; ura3-52; trp1; his4; sir3; rme</i>	Brake <i>et al.</i> (1984)
BJ5464	<i>MATα; ura3-52; trp1; leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1 .6R; can1; GAL</i>	Y.G.S.C Berkeley, California
HMSF176	<i>MATα; sec18-1</i>	R. Schekman
DC14	<i>MATα; his1</i>	J. Hicks
DC17	<i>MATα; his1</i>	J. Hicks
CHY01	<i>MATα; leu2-3,112; his4; trp1; sec18-1</i>	JRY188 x HMSF176
CHY02	<i>MATα; leu2-3,112; ura3-52::pCH13; trp1; his4; sir3; rme</i>	This study

CHY03	<i>MATα; leu2-3,112; ura3-52::pCH14; trp1; his4; sir3; rme</i>	This study
CHY04	<i>MATα; leu2-3,112; ura3-52::pCH15; trp1; his4; sir3; rme</i>	This study
CHY05	<i>MATα; leu2-3,112; ura3-52::pCH16; trp1; his4; sir3; rme</i>	This study
CHY06	<i>MATα; leu2-3,112; ura3-52::pCH17; trp1; his4; sir3; rme</i>	This study
CHY07	<i>MATα; leu2-3,112; ura3-52::YIplac211; trp1; his4; sir3; rme</i>	This study

Table A.3. Source of plasmids used in this study

YCplac22	<i>TRP1</i> yeast centromeric plasmid	Gietz <i>et al.</i> (1988)
YEplac112	<i>TRP1</i> yeast episomal plasmid	Gietz <i>et al.</i> (1988)
YIplac211	<i>URA3</i> yeast integrative plasmid	Gietz <i>et al.</i> (1988)
YEpGAL	Yeast expression vector containing GAL1/10 promoter	A. Boyd
pKpra	Protein A fusion vector	A. Boyd
pAX 11, 12 and 13	Protein A fusion vectors	Zueco and Boyd (1992)
pEX 11, 12 and 13	β -galactosidase fusion vectors	Kusters <i>et al.</i> (1989)
pCXJKan-1	<i>URA3</i> plasmid containing the Kan-r gene on a 1.3 kbp <i>SalI</i> cassette	M. Stark

pYJS50	<i>LEU2</i> , YE _p plasmid carrying a MF α 1-bla gene fusion	A. Boyd
pSEY8	Plasmid containing the complete ORF of the <i>SEC18</i> gene on a 3.0 kbp <i>Bam</i> HI/ <i>Hind</i> III cassette.	S. Emr
pK18	Derived from pUC18; Km ^R replaces Amp ^R .	R.D. Pridmore (1987)
YDpU	<i>URA3</i> containing plasmid	Berben <i>et al.</i> (1991)

Table A.4. Plasmids constructed in this study

Construct	Description
pKpra2	The plasmid pKpra was digested with <i>EcoRI/BamHI</i> and ligated with two annealed oligonucleotides 5'AAT TCTAATAACTCGAGC-3' and 5'-GATTATTGAGCT CGCTAG-3'. This deletes the <i>BamHI</i> site from the original plasmid inserting two stop codons TAA,TAA(**) and introducing a <i>XhoI</i> site which creates a <i>HindIII</i> _____** <i>XhoI</i> Pra 'cassette'.
M13SEC18	The complete <i>SEC18</i> gene was excised from the plasmid pSEY8 as a 3.0 kbp <i>ScaI/HindIII</i> fragment and ligated into the plasmid pK18 which had been digested with <i>HincII/HindIII</i> . The <i>SEC18</i> gene was then excised from pK18 as a <i>BamHI/HindIII</i> cassette and ligated into the plasmid M13mp18 which had been digested with <i>BamHI/HindIII</i> .
M13SEC18PRA	A 0.46 kbp <i>HindIII/XhoI</i> fragment from pKpra2 was treated with Klenow giving a blunt ended fragment. This fragment was ligated into M13SEC18 digested with <i>FspI</i> to produce the intermediate vector M13SEC18PRA.
YCpGAL	The GAL1/10 promoter was excised from pBM150 as a 0.685kbp <i>EcoRI/BamHI</i> fragment and ligated

into the corresponding sites in YCplac22.

YCpKan-1

The *Kan-r* gene was excised from pCXJKan-1 as a 1.3 kbp *SalI* fragment and ligated into YCplac22 which had been digested with *SalI*.

pCH1

The *SEC18pra* fusion gene was excised from M13*SEC18pra* as a 3.5kbp *BamHI/XhoI* fragment and ligated into the yeast plasmid YCplac22 which had been digested with *BamHI/SalI*.

pCH2

Same as for pCH1 except recipient plasmid was YCplac112

pCH3

Same as for pCH1 except recipient plasmid was YCpGAL

pCH4

Same as for pCH1 except recipient plasmid was YEpGAL

pCH5 and pCH6

The plasmids YCpGAL and YEpGAL were digested with *BamHI/SalI* and ligated to the two oligonucleotides 5'-GATCAAAATGTCTCCA-3' and 5'-AGCTTGGAGACATTTT-3' which contain *BamHI/HindIII* cohesive ends and an ATG in the correct reading frame to allow the expression of Pra from the *GAL1* promoter. The 0.46kbp *HindIII/XhoI* fragment from pKpra2 was ligated into this partial

ligation.

- pCH7 The *SEC18* gene was cloned as a *Bam*HI/*Hind*III fragment from the vector pSEY8 into the *Bam*HI/*Hind*III sites of YCpGAL.
- pCH7A The *SEC18* gene was cloned as a *Bam*HI/*Hind*III fragment from the vector pSEY8 into the *Bam*HI/*Hind*III sites of YEplac211.
- pCH8, 9, 10, 11 and 12 Mutated *SEC18* genes were excised as *Bam*HI/*Hind*III fragments and ligated into the plasmid YCpGAL which had been previously digested with *Bam*HI/*Hind*III.
- pCH13, 14, 15, 16 and 17 The GAL*SEC18* cassette was digested as an *Eco*RI/*Hind*III fragment from the corresponding plasmids pCH8, 9, 10, 11 and 12 and ligated into the *Eco*RI/*Hind*III sites of the vector YIplac211.
- pCH18 A 0.85 kbp fragment of the *SEC18* gene was digested from the vector pSEY8 as a *Nru*I/*Hind*III fragment which was then Klenow-treated to give a *Nru*I/[*Hind*III] fragment. This fragment was ligated into the vector pKpra which had been digested with *Sma*I.
- pCH19 A 0.8kbp fragment of the *SEC18* gene was excised as

an [*Ava*I]/*Hae*III fragment and ligated into the vector pEX12 which had been digested with *Sma*I.

pCH20

The two oligonucleotides 5'-CGGGATCCACTTCTTCCCACAAGGAAGTAGC-3' and 5'-CGGGA TCCAAGCTTGTATATATGTATGTCGCGTAAGTATATGCC-3' were used to amplify a 0.86 kbp fragment of the *SEC17* gene from yeast genomic DNA using PCR. This fragment of *SEC17* gene encodes all the Sec17p sequence except the first 11 amino acids. This fragment was cloned as a *Bam*HI fragment into the vector pAX11 which had previously been digested with *Bam*HI to produce an in frame Sec17pra fusion protein.

pCH21

The *SEC17* gene was excised from pCH20 as a *Bam*HI fragment and ligated into the vector pEX11 which had been digested with *Bam*HI to give a *SEC17* β -galactosidase fusion gene.

pCH22

The two oligonucleotides 5'CGGGATCCGGCTGCC TTTAATTGTTATTCTTTTCC-3' and 5'-CGGGATC CAAGCTTGTATATATGTATGTCGCGTAAGTATA TGCC-3' were used to amplify a 1.14 kbp full length *SEC17* gene using PCR. This fragment was digested with *Bam*HI/*Hind*III and cloned into the corresponding sites in the vector YCplac22.

pCH22A	Same as for pCH22 except the recipient plasmid was YEplac112.
pCH23	A 1.4 kbp fragment was excised from the plasmid pCH7 as a <i>Bam</i> HI/ <i>Bsm</i> I cassette (N region) and ligated into the plasmid pCH12 which had previously been digested with <i>Bam</i> HI/ <i>Bsm</i> I.
pCH24	A 0.75 kbp fragment was excised from the plasmid pCH7 as a <i>Bsm</i> I/ <i>Nru</i> I cassette (M region) and ligated into the plasmid pCH12 which had previously been digested with <i>Bsm</i> I/ <i>Nru</i> I.
pCH25	A 0.85 kbp fragment was excised from the plasmid pCH7 as a <i>Nru</i> I/ <i>Hind</i> III cassette (C region) and ligated into the plasmid pCH12 which had previously been digested with <i>Nru</i> I/ <i>Hind</i> III.

Within the above table the use of [] depicts that the restriction site has been treated with the large fragment of DNA polymerase I (Klenow) which fills-in 3' recessed ends giving blunt ends to restricted fragments.

Bibliography

Aalto, M.K., Ronne, H., and Keranen, S. (1993) Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. *EMBO J.* 12 4095-4104.

Aalto, M.K., Keranen, S., and Ronne, H. (1992) A family of proteins involved in intracellular transport. *Cell* 68 181-182.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1989) *Molecular biology of the cell* (second edition). Garland publishing, Inc. New York and London.

Anderson, I., and Denny, J.B. (1992) Protein translocation in the endoplasmic reticulum. Ultraviolet light induces the non-covalent association of nascent peptides with translocon proteins. *J. Biol. Chem.* 267 23916-23921.

Ausubel, F.M., Bret, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) *Current protocols in molecular biology.* 1 Massachusetts General Hospital. Harvard Medical School.

Balch, W.E., Dunphy, W.G., Braell, W.A., and Rothman, J.E. (1984) (Part 1) Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of *N*-Acetylglucosamine. *Cell* 39 405-416.

Balch, W.E., Glick, B.S., and Rothman, J.E. (1984) (Part 2) Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell* 39 525-536.

Banta, L.M., Robinson, J.S., Klionsky, D.J., and Emr, S.D. (1988) Organelle assembly in yeast : Characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell Biol.* 107 1369-1383.

Barlowe, C., and Schekman, R. (1993) *SEC12* encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature* 365 347-349.

Baumert, M., Maycox, P.R., Navone, F., Camilli, P.D, and Jahn, R. (1989) Synaptobrevin : An integral membrane protein of 18 000 daltons present in small synaptic vesicles of rat brain. *EMBO J.* 8 379-384.

Becherer, K.A., and Jones, E.W. (1992) EMBO Databank Rel., 31 M90395.

Beckers, C.J.M., Block, M.R., Glick, B.S., Rothman, J.E. and Balch, W.E. (1989) Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature* 339 397-398.

Bennet, M.K., Calakos, N., and Scheller, R.H. (1992) Syntaxin: A synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* 257 255-259.

Bennet, M.K., Garcia-Arrearas, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuila, C.D., and Scheller, R.H. (1993) The syntaxin family of vesicular transport receptors. *Cell* 74

Bennett, M.K.M and Scheller, R.H. (1993) The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci.* 90 2559-2563.

Berben, G., Dumont, J., Gilliquet, V., Bolle, P-A., and Hilger, F. (1991) The YDp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for *Saccharomyces cerevisiae*. *Yeast* 7 475-477.

Berstein, H.D., Poritz, M.A., Strub, K., Hoken, P.J., Brenner, S., and Walter, P. (1989) Model for signal sequence recognition from amino-acid sequence of 54K subunit recognition particle. *Nature* 340 482-486.

Blasi, J., Chapman, E.R., Link, E., Binz, T., Yamasaki, S., Niemann, H., and Jahn, R. (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP 25. *Nature* 365 160-163.

Block, M.R., Glick, B.S., Wilcox, C.A., Wieland, F.T., and Rothman, J.E. (1988) Purification of an N-ethylmaleimide-sensitive protein catalysing vesicular transport. *Proc. Natl. Acad. Sci.* 85 7852-7856.

Bowser, R., Muller, H., Govindan, B., and Novick, P. (1992) Sec8p and Sec15p are components of a plasma membrane-associated 19.5S particle that may function downstream of Sec4p to control exocytosis. *J. Cell Biol.* 118 1041-1056.

Braell, W.A., Balch, W.E., Dobbertin, D.C., and Rothman, J.E. (1984) (Part 2) The glycoprotein that is transported between successive compartments of the Golgi in a cell-free system resides in stacks of the cisternae. *Cell* 39 511-524.

Brake, A.J., Merryweather, J.P., Coit, D.G., Heberlein, U.A., Masiarz, F.R., Mullenbach, G.T. Urdea, M.S., Valenzuela, P., and Barr, P.J. (1984) α -factor directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* 81 4642-4646.

Bryant, N.J., and Boyd, A. (1993) Immunoisolation of Kex2p-containing organelles from yeast demonstrates colocalisation of the three processing proteinases to a single compartment. *J. Cell Sci.* 106 815-822.

Burton, J., Roberts, D., Montaldi, M., Novick, P., and Camilli, P. (1993) A mammalian guanine-nucleotide-releasing protein enhances function of yeast secretory protein Sec4. *Nature* 361 464-467.

Bussey, H. (1988) Proteases and the processing of precursors to secreted proteins in yeast. *Yeast* 4 17-26.

Chappell, T.G., and Warren, G. (1989) A galactosyltransferase from the fission yeast *Schizosaccharomyces pombe*. *J. Cell Biol.* 109 2693-2702.

Clary, D.O., Griff, I.C., and Rothman, J.E. (1990) SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in

animals and yeast. *Cell* 61 709-721.

Connolly, T., Collins, P., Gilmore, R. (1989) Access of proteinase K to partially translocated nascent polypeptides in intact and detergent solubilized membranes. *J. Cell Biol.* 108 299-307.

Cooper, A., and Bussey, H. (1992) Yeast Kex1p is a Golgi-associated membrane protein: Deletions in a cytoplasmic targeting domain result in mislocalization to the vacuolar membrane. *J. Cell Biol.* 119 1459-1468.

Cunningham, K.W., and Wickner, W.T. (1989) Yeast KEX2 protease and mannosyltransferase I are localized to distinct compartments of the secretory pathway. *Yeast* 5 25-33.

Dascher, C., Ossig, R., Gallwitz, D., and Schmitt, H.D. (1991) Identification and structure of four yeast genes (*SLY*) that are able to suppress the functional loss of *YPT1*, a member of the *RAS* superfamily. *Mol. and Cell. Biol.* 11 872-885.

Dean, N. and Pelham, H.R.B. (1990) Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.* 111 369-377.

Deshaies, R.J., Sanders, S.L., Feldheim, D.A. and Schekman, R. (1991) Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. *Nature* 349 806-808.

Diaz, R., Mayorga, L.S., Weidman, P.J., Rothman, J.E. and Stahl, P.D. (1989) Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature* 339 398-400.

Donaldson, J.G., Kahn, R.A., Lippincot-Schwartz, J., and Klausner, R.D. (1991) Binding of ARF and β -COP to Golgi membranes: possible regulation by a trimeric G protein. *Science* 254 1197-1199.

Dunphy, W.G., and Rothman, J.E. (1985) Compartmental organization of the Golgi stack. *Cell* 42 13-21.

Eakle, K.A., Bernstein, M., and Emr, S.D. (1988) Characterization of a component of the yeast secretion machinery: Identification of the *SEC18* gene product. *Mol. and Cell. Biol.* 8 4098-4109.

Echols, H., Lu, C., and Burgers, P.M.J. (1983) Mutator strains of *Escherichia coli*, *mutD* and *dnaQ*, with defective exonucleolytic editing by DNA polymerase III holoenzyme. *Proc. Natl. Acad. Sci.* 80 2189-2192.

Elferink, L.A., Trimble, W.S., and Scheller, R.H. (1988) Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. *J. Biol. Chem.* 264 11061-11064.

Erdmann, R., Wiebel, F.F., Flessau, A., Rytka, J., Beyer, A., Frohlich, K-U., and Kunau, W-H. (1991) *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell* 64 499-510.

Esmon, B., Novick, P., and Schekman, R. (1981)

Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell* 25 451-460.

Franzusoff, A., and Schekman, K. (1989) Functional

compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO J.* 8 2695-2702.

Frohlich, K-U., Fries, H-W., Rudiger, M., Erdmann, R.,

Botstein, D., and Mecke, D. (1991) Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J. Cell Biol.* 114 443-453.

Fuller, R.S., Stearne, R.E., and Thorner, J. (1988) Enzymes

required for yeast prohormone processing. *Annu. Rev. Physiol.* 50 345-362.

Gallwitz, D., Donath, C., and Sander, C. (1983) A yeast gene

encoding a protein homologous to the human *c-has/bas* proto-oncogene product. *Nature* 306 704-707.

Gething, M.J., McCammon, K., and Sambrook, J. (1986)

Expression of wild-type and mutant forms of Influenza hemagglutinin; the role of folding in intracellular transport. *Cell* 46 939-950.

Gething, M.J., and Sambrook, J. (1992) Protein folding in the cell.

Nature 355 33-45.

Gietz, D., Jean, A.S., Woods, R.A., and Schiestl, R.H.
(1991a) Improved method for high efficiency transformation of intact yeast cells. *Nuc. Acids Res.* 6 1425.

Gietz, R.D., and Sugino, A. (1988b) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 74 527-534.

Gilmore, R., Walter, P., and Blobel, G. (1982a) Protein translocation across the endoplasmic reticulum. I Detection in the microsomal membrane of a receptor for the signal recognition particle. *J. Cell Biol.* 95 463-469.

Gilmore, R., Walter, P., and Blobel, G. (1982b) Protein translocation across the endoplasmic reticulum. II Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* 95 470-477.

Glick, B.S., and Rothman, J.E. (1987) Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature* 326 309-312.

Gorlich, D., Hartmann, E., Prehn, S., and Rapoport, T.A.
(1992) A protein of the endoplasmic reticulum involved in early polypeptide translocation. *Nature* 357 47-52.

Goud, B., Salminen, A., Walworth, N.C., and Novick, P.J.
(1988) A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell* 53 753-768.

Gough, J.A., and Murray, N.E. (1983) Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* 166 1-19.

Graham, T.R. and Emr, S.D. (1991) Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. *J. Cell Biol.* 114 207-218.

Griff, I.C., Schekman, R., Rothman, J.E., and Kaiser, C.A. (1992) The yeast *SEC17* gene product is functionally equivalent to mammalian α - SNAP protein. *J. Biol. Chem.* 267 12106-12115.

Hann, B.C., Stirling, C.J., and Walter, P. (1992) *SEC65* gene product is a subunit of the yeast signal recognition particle required for its integrity. *Nature* 356 532-533.

Hann, B.C., and Walter, P. (1991) The signal recognition particle in *S. cerevisiae*. *Cell* 67 131-144.

Hardwick, K.G., Boothroyd, J.C., Rudner, A.D., and Pelham, H.R.B. (1992) Genes that allow yeast cells to grow in the absence of the HDEL receptor. *EMBO J.* 11 4187-4195.

Hardwick, K.G., and Pelham, H.R.B. (1992) *SED5* encodes a 39kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.* 119 513-521.

Hata, Y., Slaughter, C.A., and Sudhof, T.C. (1993) Synaptic vesicle fusion complex contains *unc-18* homologue bound to syntaxin. *Nature* 366 347-351.

Helms, J.B., Palmer, D.J., and Rothman, J.E. (1993) Two distinct populations of ARF bound to Golgi membranes. *J. Cell Biol.* 121 751-760.

Herskowitz, I. (1987) Functional inactivation of genes by dominant negative mutations. *Nature* 329 219-222.

Hiebsch, R.R., and Wattenberg, B.W. (1992) Vesicle fusion in protein transport through the Golgi does not involve long-lived prefusion intermediates. A reassessment of the kinetics of transport as measured by glycosylation. *Biochemistry* 31 6111-6118.

High, S., and Dobberstein, B. (1991) The signal sequence interacts with the methionine-rich domain of the 54kD protein of signal recognition particle. *J. Cell Biol.* 113 229-233.

Hoffman, C.S., Winston, F. (1987) A ten-minute DNA preparation from yeast efficiently releases autosomous plasmids for transformation of *Escherichia coli*. *Gene* 57 267-272.

Hosobuchi, M., Kreis, T., and Schekman, R. (1992) *SEC21* is a gene required for ER to Golgi protein transport that encodes a subunit of a yeast coatomer. *Nature* 360 603-605.

Huttner, W.B. (1993) Snappy exocytosis. *Nature* 365 104-105.

Kahn, R.A., and Gilman, A.G. (1984) Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J. Biol. Chem.* 259 6228-34.

Kaiser, C.A., and Schekman, R. (1990) Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* 61 723-733.

Kelly, R.B. (1993) Much ado about docking. *Curr. Biol.* 3 474-476.

Koshland, D., Kent, J.C. and Hartwell, L.H. (1985) Genetic analysis of the mitotic transmission of minichromosomes. *Cell* 40 393-403.

Kukuruzinska, M.A., Bergh, M.L.E., and Jackson, B.J. (1987) Protein glycosylation in yeast. *Annu. Rev. Biochem.* 56 915-944.

Kurjan, J., and Herskowitz, I. (1982) Structure of a yeast pheromone gene (*MF α*): A putative α -factor precursor contains four tandem copies of mature α -factor. *Cell* 30 933-943.

Kusters, J.G., Jager, E.J., and van der Zeijst, B.A.M. (1989) Improvement of the cloning linker of the bacterial expression vector pEX. *Nucl. Acids Res.* 17 8007.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 680-685.

Lewis, M.J., and Pelham, H.R.B. (1990) A human homologue of the yeast HDEL receptor. *Nature* 348 162-163.

Lewis, M.J., Sweet, D.J., and Pelham, H.R.B. (1990) The *ERD2* gene determines the specificity of the luminal ER protein retention system. *Cell* 61 1359-1363.

Lowenadler, B., Nilsson, B., Abrahmsen, L., Moks, T., Ljungqvist, L., Holmgren, E., Paleus, S., Josephson, S., Philipson, L. and Uhlen, M. (1986) Production of specific antibodies against protein A fusion proteins. *EMBO J.* 5 2393-2398.

Lutcke, H., High, S., Romisch, K., Ashford, A.J., and Dobberstein, B. (1992) Methionine-rich domain of the 54kDa subunit of signal recognition particle is sufficient for the interaction with signal sequences. *EMBO J.* 11 1543-1551.

Lian, J.P., and Ferro-Novick, S. (1993) Bos1p, an integral membrane protein of the endoplasmic reticulum to Golgi transport vesicles, is required for their fusion competence. *Cell* 73 735-745.

Lowenadler, B., Nilsson, B., Abrahmsen, L., Moks, T., Ljungqvist, L., Holmgren, E., Paleus, S., Josephson, S., Philipson, L., and Uhlen, M. (1986) Production of specific antibodies against protein A fusion proteins. *EMBO J.* 5 2393-2398.

Malhotra, V., Orci, L., Glick, B.S., Block, M.R. and Rothman, J.E. (1988) Role of an *N*-ethylmaleimide-sensitive transport

component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell* 54 221-227.

Matsui, Y., Kikuchi, A., Kondo, J., Hishida, T., Teranishi, T., and Takai, Y. (1988) Nucleotide and deduced amino acid sequences of a GTP-binding protein family with molecular weights of 25,000 from bovine brain. *J. Biol. Chem.* 263 11071-11074.

Mellman, I., and Simons, K. (1992) The Golgi complex: *in vitro* veritas ? *Cell* 68 829-840.

Moya, M., Roberts, D., and Novick, P. (1993) *DSS4-1* is a dominant suppressor of *sec4-8* that encodes a nucleotide exchange protein that aids Sec4p function. *Nature* 361 460-463.

Muhlrad, D., Hunter, R., and Parker, R. (1992) A rapid method for localized mutagenesis of yeast genes. *Yeast* 8 79-82.

Munro, S., and Pelham, H.R.B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48 899-907.

Nakano, A., and Muramatsu, M. (1989) A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* 109 2677-2691.

Newman, A.P., and Ferro-Novick, S. (1987) Characterization of new mutants in the early part of the yeast secretory pathway isolated by a [³H] mannose suicide selection. *J. Cell Biol.* 105 1587-1594.

Newman, A.P., Groesch, M.E., and Ferro-Novick, S. (1992) Bos1p, a membrane protein required for ER to Golgi transport in yeast, co-purifies with the carrier vesicles and with Bet1p and the ER membrane. *EMBO J.* 11 3609-3617.

Nieto, A., Sanz, P., Sentandreu, R., and Del Castillo Agudo, L. (1993) Cloning and characterization of the *SEC18* gene from *Candida albicans*. *Yeast* 9 875-887.

Novick, P., and Brennwald, P. (1993) Friends and family: The role of the Rab GTPases in vesicular traffic. *Cell* 75 597-601.

Novick, P., Ferro, S., and Schekman, R. (1981) Order of events in the yeast secretory pathway. *Cell* 25 461-469.

Novick, P., Field, C., and Schekman, R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21 205-215.

Novick, P. and Schekman, R. (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* 76 1858-1862.

Ogg, S.C., Poritz, M.A. and Walter, P. (1992) Signal recognition particle receptor is important for cell growth and protein secretion in *Saccharomyces cerevisiae*. *Mol. Biol. of the Cell* 3 895-911.

- Oka, T., Nishikawa, S-I., and Nakano, A.** (1991) Reconstitution of GTP binding Sar1 protein function in ER to Golgi transport. *J. Cell Biol.* *114* 671-679.
- Orci, L., Malhotra, V., Amherdt, M., Serafini, T. and Rothman, J.E.** (1989) Dissection of a single round of vesicular transport: Sequential intermediates for intercisternal movement in the Golgi stack. *Cell* *56* 357-368.
- Ossig, R., Dascher, C., Trepte, HH., Schmitt, H.D., and Gallwitz, D.** (1991) The yeast *SLY* gene products, suppressors of defects in the essential GTP-binding Ypt1 protein, may act in endoplasmic reticulum-to-Golgi transport. *Mol. and Cell. Biol.* *11* 2980-2993.
- Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingslay, M., Bloom, F.E., and Wilson, M.C.** (1989) The identification of a novel synaptosomal-associated protein SNAP25, differentially expressed by neuronal subpopulations. *J. Cell Biol.* *109* 3039-3052.
- Palade, G.** (1975) Intracellular aspects of the process of protein synthesis. *Science* *189* 347-358.
- Pelham, H.R.B.** (1988) Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.* *7* 913-918.
- Pelham, H.R.B., Hardwick, K.G., and Lewis, M.J.** (1988) Sorting of soluble ER proteins in yeast. *EMBO J.* *7* 1757-1760.

- Peters, J.M., Walsh, M.J., and Franke, W.W.** (1990) An abundant and ubiquitous homo-oligomeric ring-shaped ATPase particle related to the putative vesicle fusion proteins Sec18p and NSF. *EMBO J.* 9 1757-1767.
- Pfanner, N., Glick, B.S., Arden, S.R. and Rothman, J.E.** (1990) Fatty acylation promotes fusion of transport vesicles with Golgi cisternae. *J. Cell Biol.* 110 955-961.
- Preuss, D., Mulholland, J., Franzusoff, A., Segev, N., and Botstein, D.** (1992) Characterization of the *Saccharomyces* Golgi complex through the cell cycle by immunoelectron microscopy. *Mol. Biol. Cell* 3 789-803.
- Pridmore, A.D.** (1987) New and versatile cloning vectors with kanamycin resistance marker. *Gene* 56 309-312.
- Protopopov, V., Govindan, B., Novick, P., and Gerst, J.E.** (1993) Homologs of the Synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell* 74 855-861.
- Rapoport, T.A.** (1991) Protein transport across the endoplasmic reticulum membrane: facts, models, mysteries. *FASEB J.* 5 2792-2798.
- Redding, K., Holcomb, C., and Fuller, R.S.** (1991) Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 113 527-

Rexach, M.F. and Schekman, R.W. (1991) Distinct biochemical requirements for budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* *114* 219-229.

Riezman, H. (1985) Endocytosis in yeast: Several of the yeast secretory mutants are defective in endocytosis. *Cell* *40* 1001-1009.

Roberts, C.J., Nothwehr, S.F., and Stevens, T.H. (1992) Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. *J. Cell Biol.* *119* 69-83.

Romisch, K., and Schekman, R. (1992) Distinct processes mediate glycoprotein and glycopeptide export from the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* *89* 7227-7231.

Rose, M.D., Novick, P., Thomas, J.H., Botstein, D., and Fink, G.R. (1987) A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromeric-containing shuttle vector. *Gene* *60* 237-243.

Rose, M., and Winston, F. (1984) Identification of a Ty insertion within the coding sequence of the *S.cerevisiae* *URA3* gene. *Mol. Gen. Genet.* *193* 557-560.

Salama, N.R., Yeung, T. and Schekman, R.W. (1993) The Sec13p complex and reconstitution of vesicle budding from the ER with purified cytosolic proteins. *EMBO J.* *12* 4073-4082.

Salminen, A., and Novick, P.J. (1987) A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell* 49 527-538.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual, 2nd Edition. Cold Spring Harbor Laboratory press. Cold Spring Harbor, New York.

Sanders, S.L., Whitfield, K.M., Vogel, J.P., Rose, M.D., and Schekman, R.W. (1992) Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell* 69 353-365.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci.* 74 5463.

Sawyer, L., Fothergill-Gilmore, L.A. and Russell, G. (1986) The predicted secondary structure of enolase. *Biochem. J.* 236 127-130.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C. (1993) Tetanus and botulinum neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* 359 832-835.

Schleifer, L.S., Kahn, R.A., Hanski, E., Northup, J.K., Sternweis, P.C., Gilman, A.G. (1982) Requirements for Cholera Toxin dependent ADP ribosylation of the purified regulatory component of adenylate cyclase. *J. Biol. Chem.* 257 20-23.

Schultz, L.D., Hofmann, K.J., Mylin, L.M., Montgomery, D.L., Ellis, R.W. and Hopper, J.E. (1987) Regulated overproduction of the *GAL4* gene product greatly increases expression from galactose-inducible promoters on multi-copy expression vectors in yeast. *Gene* 61 123-133.

Segev, N., Mulholland, J., and Botstein, D. (1988) The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell* 52 915-924.

Semenza, J.C., Hardwick, K.G., Dean, N., and Pelham, H.R.B. (1990) *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* 61 1349-1357.

Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R.A., and Rothman, J.E. (1991) ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: A novel role for a GTP-binding protein. *Cell* 67 239-253.

Sollner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993b) A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75 409-418.

Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993a) SNAP receptors implicated in vesicle targeting and fusion.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98 503.

Stearns, T., Willingham, M.C., Botstein, D., Kahn, R.A. (1990) ADP-ribosylation factor is functionally and physically associated with the Golgi complex. *Proc. Nat. Acad. Sci.* 87 1238-1242.

Stenbeck, G., Harter, C., Brecht, A., Herrmann, D., Lottspeich, F. Orci, L., and Wieland, F.T. (1993) β' - COP a novel subunit of coatomer. *EMBO J.* 12 2841-2845.

Stenbeck, G., Schreiner, R., Herrmann, D., Auerbach, S., Lottspeich, F., Rothman, J.E., and Wieland, F.T. (1992) γ -COP, a coat subunit of non-clathrin-coated vesicles with homology to Sec21p. *FEBS* 314 195-198.

Stevens, T., Esmon, B., and Schekman, R. (1982) Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell* 30 439-448.

Stirling, C.J., and Hewitt, E.W. (1992) The *S. cerevisiae* SEC65 gene encodes a component of yeast signal recognition particle with homology to human SRP19. *Nature* 356 534-537.

Stirling, C.J., Rothblatt, J., Hosobuchi, M., Deshaies, R. and Schekman, R. (1992) Protein translocation mutants defective in the

insertion of integral membrane proteins into the endoplasmic reticulum. *Mol. Biol. of the Cell* 3 129-142.

Sweet, D.J. and Pelham, H.R.B. (1993) The *TIP1* gene of *Saccharomyces cerevisiae* encodes an 80kDa cytoplasmic protein that interacts with the cytoplasmic domain of Sec20p. *EMBO J.* 12 2831-2840.

Tagaya, M., Wilson, D.W., Brunner, M., Arango, N. and Rothman, J.E. (1993) Domain structure of an *N*-Ethylmaleimide-sensitive fusion protein involved in vesicular transport. *J. Biol. Chem.* 268 2662-2666.

Takizawa, P.A., and Malhotra, V. (1993) Coatamers and SNAREs in promoting membrane traffic. *Cell* 75 593-596.

Touchot, N., Chardin, P., and Tavitian, A. (1987) Four additional members of the *ras* gene superfamily isolated by an oligonucleotide strategy: Molecular cloning of *YPT*-related cDNAs from a rat brain library. *Proc. Natl. Acad. Sci.* 84 8210-8214.

Van der Aart, Q.J.M. (1994) *EMBO Databank Rel.*, 38 X76294.

Von Heijine, G. (1985) Signal sequences: The limits of variation. *J. Mol. Biol.* 184 99-105.

Wada, Y., Kitamoto, K., Kanbe, T., Tanaka, K., and Anraku, Y. (1990) The *SLP 1* gene of *Saccharomyces cerevisiae* is essential for vacuolar morphogenesis and function. *Mol. cell Biol.* 10 2214-

Walter, P., and Blobel, G. (1980) Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. *P.N.A.S.* 77 7112-7116.

Waters, M.G., Serafini, T., and Rothman, J.E. (1991) 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature* 349 248-251.

Wattenberg, B.W., and Rothman, J.E. (1986) Multiple cytosolic components promote intra-Golgi protein transport. *J. Biol. Chem.* 261 2208-2213.

Wattenberg, B.W., Balch, W.E., and Rothman, J.E. (1986) A novel prefusion complex formed during protein transport between Golgi cisternae in a cell-free system. *J. Biol. Chem.* 261 2202-2207.

Wattenberg, B.W., Hiebsch, R.R., LeCureux, L.W., and White, M.P. (1990) Identification of a 25kD protein from yeast cytosol that operates in a prefusion step of vesicular transport between compartments of the Golgi. *J. Cell Biol.* 110 947-954.

Wattenberg, B.W., Raub, T.J., Hiebsch, R.R. and Weidman, P.J. (1992) The activity of Golgi transport vesicles depends on the presence of the *N*-ethylmaleimide-sensitive factor (NSF) and a soluble NSF attachment protein (α SNAP) during vesicle formation. *J. Cell Biol.* 118 1321-1332.

Weidman, P.J., Melancon, P., Block, M.R., and Rothman, J.E. (1989) Binding of an *N*-Ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. *J. Cell Biol.* 108 1589-1596.

Whiteheart, S.W., Brunner, M., Wilson, D.W., Wiedmann, M., and Rothmen, J.E. (1992) Soluble *N*-Ethylmaleimide-sensitive fusion attachment proteins (SNAPs) bind to a multi-SNAP receptor complex in Golgi membranes. *J. Biol. Chem.* 267 12239-12243.

Whiteheart, S.W., Griff, I.C., Brunner, M., Clary, D.O., Mayer, T., Buhrow, S.A., and Rothman, J.E. (1993) SNAP family of NSF attachment proteins includes a brain-specific isoform. *Nature* 362 353-355.

Wieland, F.T., Gleason, M.L., Serafini, T.A. and Rothman, J.E. (1987) The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell* 50 289-300.

Wilson, D.W., Whiteheart, S.W., Wiedmann, M., Brunner, M., and Rothman, J.E. (1992) A multisubunit particle implicated in membrane fusion. *J. Cell Biol.* 117 531-538.

Wilson, D.W., Wilcox, C.A., Flynn, G.C., Chen, E., Kuang, W.J., Henzel, W.J., Block, M.R., Ullrich, A. and Rothman, J.E. (1989) A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* 339 355-359.

Yoshihisa, T., Barlowe, C., and Schekman, R. (1993)

Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science* 259 1466-1468.

Zerial, M., and Stenmark, H. (1993) Rab GTPases in vesicular

transport. *Curr. Opin. Cell Biol.* 5 631-620.

Zueco, J., and Boyd, A. (1992) Protein A fusion vectors for use in

combination with pEX vectors in the production and affinity purification of specific antibodies. *Gene* 121 181-182.

Zopf, D., Bernstein, H.D., Johnson, A.E., and Walter, P.

(1990) The methionine-rich domain of the 54kD protein subunit of the signal recognition particle contains an RNA binding site and can be crosslinked to a signal sequence. *EMBO J.* 9 4511-4517.